Study Title

Low Dicyclopentadiene Resin Oil: Bacterial Reverse Mutation Test

Test Substance

Low DCPD Resin Oil

Authors

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Study Completion Date

16 June 2004

Testing Facility

BioReliance
9630 Medical Center Drive
Rockville, MD 20850
for
American Chemistry Council
Olefins Panel
1300 Wilson Boulevard
Arlington, VA 22209
under contract with
E.I. du Pont de Nemours and Company
DuPont Haskell Laboratory
Newark, DE 19714-0050

BioReliance Study Number

AA75HB.502.BTL

Work Request Number

14295

Haskell Number

H-25429

Service Code

500

American Chemistry Council Reference Number

OLF-92.0-HPV789-DHL

STATEMENT OF COMPLIANCE

Study No. AA75HB.502.BTL was conducted in compliance with the U.S. EPA GLP Standards 40 CFR 792 in all material aspects with the following exceptions:

The characterization of the test substance presented in Appendix D was not conducted in compliance with the above regulation.

The stability of the test substance has not been determined by the testing facility.

Analyses to determine the uniformity or concentration of the test mixtures and their stability were not performed by the testing facility or the Sponsor.

Valentine O. Wagner, III — 16 Jun 2004

Valentine O. Wagner, III, M.S.
Study Director

Date

[signature] — 16 Jun 2004

BioReliance Study Management

Date

Quality Assurance Statement

Study Title: BACTERIAL REVERSE MUTATION ASSAY

Study Number: AA75HB.502.BTL

Study Director: Valentine O. Wagner, III, M.S.

Quality Assurance performed the inspections listed below for this study. Verification of the study protocol was also performed and documented by QA. Procedures, documentation, equipment records, etc., are examined in order to assure that the study is performed in accordance with the U.S. FDA Good Laboratory Practice Regulations (21 CFR 58), the U.S. EPA GLPs (40 CFR 792 and 40 CFR 160), the UK GLP Regulations, the Japanese GLP Standard, and the OECD Principles of Good Laboratory Practice and to assure that the study is conducted according to the protocol and relevant Standard Operating Procedures.

The following are the inspection dates, phases inspected, and report dates of QA inspections of this study.

Inspect On:	29-May-03 - 29-May-03 To Study Dir 29-May-03 To Mgmt 29-May-03
Phase:	Protocol Review
Inspect On:	10-Jun-03 - 10-Jun-03 To Study Dir 10-Jun-03 To Mgmt 10-Jun-03
Phase:	Preparation of S9 mixture
Inspect On:	30-Jun-03 - 01-Jul-03 To Study Dir 01-Jul-03 To Mgmt 02-Jul-03
Phase:	Draft Report
Inspect On:	11-Mar-04 - 11-Mar-04 To Study Dir 11-Mar-04 To Mgmt 11-Mar-04
Phase:	Draft to Draft II Report
Inspect On:	14-Jun-04 - 16-Jun-04 To Study Dir 14-Jun-04 To Mgmt 16-Jun-04
Phase:	Draft to Final Report

This report describes the methods and procedures used in the study and the reported results accurately reflect the raw data of the study.

Anabelle Hernandez, B.S. — 16 Jun 2004
QUALITY ASSURANCE — DATE

CERTIFICATION

We, the undersigned, declare that this report provides an accurate evaluation of data obtained from this study.

Issued by Study Director: Valentine O. Wagner, III — 16 Jun 2004
Valentine O. Wagner III, M.S. — Date

Approved by Study Monitor: E. Maria Donner — 07-Jun-2004
E. Maria Donner, Ph.D. — Date
Senior Research Toxicologist

TABLE OF CONTENTS

Study Information

CA Index Name: Distillates (petroleum), steam-cracked, C8-12 fraction

Synonyms/Codes:
- Low DCPD Resin Oil
- Low Dicyclopentadiene Resin Oil
- Lyondell Resin Oil – 90 (LRO-90)
- Steam-cracked aromatic naptha
- ARF
- Aromatic Resin Feedstock
- H-25429

Haskell Number: 25429

CAS Registry Number: 68477-54-3

Composition: (provided by the sponsor)

0.0005 wt %	Benzene
0.0074 wt %	BHT (Butylated Hydroxytoluene)
0.01 wt %	Ethylbenzene
0.29 wt %	meta-, ortho-, and para- Xylene
0.52 wt %	Styrene
0.68 wt %	DCPD (Dicyclopentadiene)
0.92 wt %	123HEMMI (1,2,3-Trimethylbenzene)
1.8 wt %	1,3,5-Trimethylbenzene
1.47 wt %	Naphthalene
2.71 wt %	alpha-, cis-beta-, and trans-beta- Methyl Styrene
6.45 wt %	1,2,4-trimethylbenzene
8.35 wt %	Methyl Indenes (total)
13.68 wt %	Indene
17.29 wt %	meta-, ortho-, and para- Vinyl Toluene

The balance of the composition is expected to consist of other hydrocarbons with carbon range primarily 8 to 10, mainly aromatics and olefins, with some paraffins.

Physical Characteristics: Colorless - light yellow liquid

Stability: The test substance appeared to be stable under the conditions of the study; no evidence of instability was observed.

Sponsor: American Chemistry Council
1300 Wilson Boulevard
Arlington, Virginia 22209
U.S.A.

Study Initiated/Completed: May 27, 2003 / (see report cover page)

SUMMARY

The test substance, Low DCPD Resin Oil, was tested in the Low Dicyclopentadiene Resin Oil: Bacterial Reverse Mutation Test using *Salmonella typhimurium* tester strains TA98, TA100, TA1535 and TA1537 and *Escherichia coli* tester strain WP2 *uvr*A in the presence and absence of Aroclor-induced rat liver S9. The test was performed in two phases, using the plate incorporation method. The first phase, the preliminary toxicity test, was used to establish the dose-range for the mutagenicity test. The second phase, the mutagenicity test was used to evaluate the mutagenic potential of the test substance.

Ethanol (EtOH) was selected as the solvent of choice based on solubility of the test substance and compatibility with the target cells. The test substance was a soluble and clear solution in ethanol (EtOH) at approximately 500 mg/mL, the highest concentration tested.

In the preliminary toxicity test, the maximum dose tested was 5000 µg per plate; this dose was achieved using a concentration of 100 mg/mL and a 50 µL plating aliquot. The dose levels tested were 6.7, 10, 33, 67, 100, 333, 667, 1000, 3333 and 5000 µg per plate. Toxicity was observed with some conditions beginning at 3333 or at 5000 µg per plate. Precipitate was observed beginning at 3333 or at 5000 µg per plate. Based on the findings of the preliminary toxicity test, the maximum dose plated in the mutagenicity test was 5000 µg per plate.

In the mutagenicity test, no positive mutagenic response was observed. The dose levels tested were 75, 200, 600, 1800 and 5000 µg per plate. Toxicity was observed with some conditions beginning at 1800 or at 5000 µg per plate. Precipitate was observed at 5000 µg per plate with most conditions.

The results of the Low Dicyclopentadiene Resin Oil: Bacterial Reverse Mutation Test indicate that, under the conditions of this study, Low DCPD Resin Oil did not cause a positive response in either the presence or absence of Aroclor-induced rat liver S9. The study was concluded to be negative without conducting a confirmatory test because no unique metabolism requirements were known about the test substance and because no equivocal responses were observed in the test that would suggest further testing is warranted.

PURPOSE

The purpose of this study was to evaluate the mutagenic potential of the test substance by measuring its ability to induce reverse mutations at selected loci of several strains of *Salmonella typhimurium* and at the tryptophan locus of *Escherichia coli* strain WP2 *uvr*A in the presence and absence of Aroclor-induced rat liver S9. A copy of the protocol is included in Appendix B.

The study was conducted to comply with OECD Guideline 471 (Genetic Toxicology: Bacterial Reverse Mutation Test), adopted July 1997 (published February 1998), with OPPTS Guideline 870.5100 (Bacterial Reverse Mutation Test) and with the EC Commission Directive 2000/32/EC.

CHARACTERIZATION OF TEST AND CONTROL SUBSTANCES

The test substance, Low DCPD Resin Oil, was received by BioReliance on 12 May 2003 and was assigned the code number AA75HB. The test substance was supplied by E.I. du Pont de Nemours and Company as a liquid and was stored refrigerated and protected from light and air. The original container was kept blanketed with nitrogen. Test substance used in the study may be dispensed into clear glass containers and does not need to be handled in any special manner. An expiration date of 06 November 2003 was provided. Upon receipt, the test substance was stored at 2-8°C in the dark under nitrogen. Upon initial use the test substance was described as a light yellow liquid.

DuPont Haskell Laboratory of E.I. du Pont de Nemours and Company has determined the identity, strength, purity, composition or other characteristics to define the test substance.

The vehicle used to deliver Low DCPD Resin Oil to the test system was 100 % ethanol (EtOH, CAS No. 64-17-5), obtained from Pharmco Products, Inc. Test substance dilutions were prepared immediately before use and delivered to the test system at room temperature under yellow light.

The negative and positive control articles have been characterized as per the Certificates of Analysis on file with the testing facility. The stability of the negative and positive control Substances and their mixtures was demonstrated by acceptable results that met the criteria for a valid test.

Positive controls plated concurrently with the mutagenicity test are listed in the following table. All positive controls were diluted with dimethyl sulfoxide (DMSO) except sodium azide, which was diluted with water. All subdivided solutions of positive control were stored at –5 to –30°C.

Strain	S9 Activation	Positive Control	Concentration (µg/plate)
All *Salmonella* Strains	Rat	2-aminoanthracene (Aldrich Chemical Co., Inc.) Lot No. 09106PS Exp. Date 14-Sep-2005 CAS No. 613-13-8 Purity 97.4%	1.0
WP2 *uvr*A			10
TA98	None	2-nitrofluorene (Aldrich Chemical Co., Inc.) Lot No. 08708HS Exp. Date 08-Mar-2006 CAS No. 607-57-8 Purity 99%	1.0
TA100, TA1535		Sodium azide (Sigma Chemical Co.) Lot No. 098H0169 Exp. Date 05-Jan-2004 CAS No. 26628-22-8 Purity 99.8%	1.0
TA1537		9-aminoacridine (Sigma Chemical Co.) Lot No. 106F06681 Exp. Date 08-Nov-2004 CAS No. 90-45-9 Purity >97%	75
WP2 *uvr*A		Methyl methanesulfonate (Aldrich Chemical Co., Inc.) Lot No. 15526AO Exp. Date 23-Jul-2004 CAS No. 66-27-3 Purity 99.9%	1,000

To confirm the sterility of the test substance, the highest test substance dose level used in the mutagenicity test was plated on selective agar with an aliquot volume equal to that used in the test. These plates were incubated under the same conditions as the test.

MATERIALS AND METHODS

Test System

The tester strains used were the *Salmonella typhimurium* histidine auxotrophs TA98, TA100, TA1535 and TA1537 as described by Ames *et al.* (1975) and *Escherichia coli* WP2 *uvr*A as described by Green and Muriel (1976). *Salmonella* tester strains were received directly from Dr. Bruce Ames' designated distributor, Discovery Partners International, San Diego, California. *E. coli* tester strains were received from the National Collection of Industrial and Marine Bacteria, Aberdeen, Scotland.

Tester strains TA98 and TA1537 are reverted from histidine dependence (auxotrophy) to histidine independence (prototrophy) by frameshift mutagens. Tester strain TA1535 is reverted by mutagens that cause basepair substitutions. Tester strain TA100 is reverted by mutagens that cause both frameshift and basepair substitution mutations. Specificity of the reversion mechanism in *E. coli* is sensitive to base-pair substitution mutations, rather than frameshift mutations (Green and Muriel, 1976).

Overnight cultures were prepared by inoculating from the appropriate master plate or from the appropriate frozen permanent stock into a vessel containing ~50 mL of culture medium. To assure that cultures were harvested in late log phase, the length of incubation was controlled and monitored. Following inoculation, each flask was placed in a resting shaker/incubator at room temperature. The shaker/incubator was programmed to begin shaking at approximately 125 rpm at 37±2°C approximately 12 hours before the anticipated time of harvest. Each culture was monitored spectrophotometrically for turbidity and was harvested at a percent transmittance yielding a titer of approximately 10^9 cells per milliliter. The actual titers were determined by viable count tests on nutrient agar plates, and the data is on file but not presented in this report.

Metabolic Activation System

Aroclor 1254-induced rat liver S9 was used as the metabolic activation system. The S9 was prepared from male Sprague-Dawley rats induced with a single intraperitoneal injection of Aroclor 1254, 500 mg/kg, five days prior to sacrifice. The S9 was batch prepared and stored at -70°C or colder until used. Each bulk preparation of S9 was tested for its ability to metabolize 2-aminoanthracene and 7,12-dimethylbenz(a)anthracene to forms mutagenic to *Salmonella typhimurium* TA100.

The S9 mix was prepared immediately before its use and contained 10% S9, 5 mM glucose-6-phosphate, 4 mM ß-nicotinamide-adenine dinucleotide phosphate, 8 mM $MgCl_2$ and 33 mM KCl in a 100 mM phosphate buffer at pH 7.4. The Sham S9 mixture (Sham mix), containing 100 mM phosphate buffer at pH 7.4, was prepared immediately before its use. To

confirm the sterility of the S9 and Sham mixes, a 0.5 mL aliquot of each was plated on selective agar.

Solubility Test

A solubility test was conducted to select the vehicle. The test was conducted using dimethyl sulfoxide (DMSO) and ethanol (EtOH). The test substance was tested to determine the vehicle that permitted preparation of the highest soluble or workable stock concentration, up to 500 mg/mL for organic solvents. Although the test substance was soluble in both dimethyl sulfoxide and ethanol, ethanol was selected based on the Sponsor's preference for ethanol.

Preliminary Toxicity Test

The preliminary toxicity test was used to establish the dose-range over which the test substance would be tested. Vehicle and ten dose levels of the test substance were plated, one plate per dose, with overnight cultures of TA98, TA100, TA1535, TA1537 and WP2 *uvr*A on selective minimal agar in the presence and absence of Aroclor-induced rat liver S9. The dose levels tested were 6.7, 10, 33, 67, 100, 333, 667, 1000, 3333 and 5000 µg per plate.

Mutagenicity Test

The mutagenicity test was used to evaluate the mutagenic potential of the test substance. Five dose levels of test substance along with appropriate vehicle control and positive controls were plated with overnight cultures of TA98, TA100, TA1535, TA1537 and WP2 *uvr*A in the presence and absence of Aroclor-induced rat liver S9. The dose levels tested were 75, 200, 600, 1800 and 5000 µg per plate. All dose levels of test substance, vehicle control and positive controls were plated in triplicate.

Plating and Scoring Procedures

The test system was exposed to the test substance via the plate incorporation methodology originally described by Ames *et al.* (1975) and updated by Maron and Ames (1983).

On the day of its use, minimal top agar, containing 0.8 % agar (W/V) and 0.5 % NaCl (W/V), was melted and supplemented with L-histidine, D-biotin and L-tryptophan solution to a final concentration of 50 µM each. Top agar not used with S9 or Sham mix was supplemented with 25 mL of water for each 100 mL of minimal top agar. For the preparation of media and reagents, all references to water imply sterile, deionized water produced by the Milli-Q Reagent Water System. Bottom agar was Vogel-Bonner minimal medium E (Vogel and Bonner, 1956) containing 1.5 % (W/V) agar. Nutrient bottom agar was Vogel-Bonner minimal medium E containing 1.5 % (W/V) agar and supplemented with 2.5 % (W/V) Oxoid Nutrient Broth No. 2 (dry powder). Nutrient Broth was Vogel-Bonner salt solution supplemented with 2.5 % (W/V) Oxoid Nutrient Broth No. 2 (dry powder).

Each plate was labeled with a code system that identified the test substance, test phase, dose level, tester strain and activation, as described in detail in BioReliance's Standard Operating Procedures.

One-half (0.5) milliliter of S9 or Sham mix, 100 µL of tester strain and 50 µL of vehicle or test substance dilution were added to 2.0 mL of molten selective top agar at 45±2°C. After vortexing, the mixture was overlaid onto the surface of 25 mL of minimal bottom agar. When plating the positive controls, the test substance aliquot was replaced by a 50 µL aliquot of appropriate positive control. After the overlay had solidified, the plates were inverted and incubated for approximately 48 to 72 hours at 37±2°C. Plates that were not counted immediately following the incubation period were stored at 2-8°C until colony counting could be conducted (less than 10 days).

The condition of the bacterial background lawn was evaluated for evidence of test substance toxicity by using a dissecting microscope. Precipitate was evaluated by visual examination without magnification. Toxicity and degree of precipitation were scored relative to the vehicle control plate using the codes shown in the following table.

Code	Description	Characteristics
1	Normal	Distinguished by a healthy microcolony lawn.
2	Slightly Reduced	Distinguished by a noticeable thinning of the microcolony lawn and possibly a slight increase in the size of the microcolonies compared to the vehicle control plate.
3	Moderately Reduced	Distinguished by a marked thinning of the microcolony lawn resulting in a pronounced increase in the size of the microcolonies compared to the vehicle control plate.
4	Extremely Reduced	Distinguished by an extreme thinning of the microcolony lawn resulting in an increase in the size of the microcolonies compared to the vehicle control plate such that the microcolony lawn is visible to the unaided eye as isolated colonies.
5	Absent	Distinguished by a complete lack of any microcolony lawn over greater than or equal to 90% of the plate.
6	Obscured by Precipitate	The background bacterial lawn cannot be accurately evaluated due to microscopic test substance precipitate.
NP	Non-Interfering Precipitate	Distinguished by precipitate on the plate that is visible to the naked eye but any precipitate particles detected by the automated colony counter total less than 10% of the revertant colony count (e.g., less than 3 particles on a plate with 30 revertants).

Code	Description	Characteristics
IP	Interfering Precipitate	Distinguished by precipitate on the plate that is visible to the naked eye and any precipitate particles detected by the automated colony counter exceed 10% of the revertant colony count (e.g., more than 3 particles on a plate with 30 revertants). These plates are counted manually.

Revertant colonies for a given tester strain and activation condition, except for positive controls, were counted either entirely by automated colony counter or entirely by hand unless the plate exhibited toxicity.

Evaluation of Results

For each replicate plating, the mean and standard deviation of the number of revertants per plate were calculated and are reported.

For the test substance to be evaluated positive, it must cause a dose-related increase in the mean revertants per plate of at least one tester strain over a minimum of two increasing concentrations of test substance. Data sets for tester strains TA1535 and TA1537 were judged positive if the increase in mean revertants at the peak of the dose response is equal to or greater than 3.0-times the mean vehicle control value. Data sets for tester strains TA98, TA100 and WP2 *uvr*A were judged positive if the increase in mean revertants at the peak of the dose response is equal to or greater than 2.0-times the mean vehicle control value.

Criteria for a Valid Test

The following criteria must be met for the mutagenicity test to be considered valid. All *Salmonella* tester strain cultures must demonstrate the presence of the deep rough mutation (*rfa*) and the deletion in the *uvr*B gene. Cultures of tester strains TA98 and TA100 must demonstrate the presence of the pKM101 plasmid R-factor. All WP2 *uvr*A cultures must demonstrate the deletion in the *uvr*A gene. All cultures must demonstrate the characteristic mean number of spontaneous revertants in the vehicle controls as follows (inclusive): TA98, 10 - 50; TA100, 80 - 240; TA1535, 5 - 45; TA1537, 3 - 21; WP2 *uvr*A, 10 - 60. To ensure that appropriate numbers of bacteria are plated, tester strain culture titers must be greater than or equal to $0.3x10^9$ cells/mL. The mean of each positive control must exhibit at least a 3.0-fold increase in the number of revertants over the mean value of the respective vehicle control. A minimum of three non-toxic dose levels is required to evaluate test data. A dose level is considered toxic if one or both of the following criteria are met: (1) A >50 % reduction in the mean number of revertants per plate as compared to the mean vehicle control value. This reduction must be accompanied by an abrupt dose-dependent drop in the revertant count. (2) At least a moderate reduction in the background lawn (background lawn code 3, 4 or 5). A copy of the Historical Negative and Positive Control Values is included in Appendix A.

Archives

At the completion of the study, all raw data, the protocol and all reports for procedures performed at BioReliance will be archived at E.I. du Pont de Nemours and Company per contractual arrangements between the American Chemistry Council and E.I. du Pont de Nemours and Company. All study materials returned to the E.I. du Pont de Nemours and Company will first be copied and the copy will be retained in the BioReliance archives for a minimum of 10 years. Unused dosing solutions were disposed of following administration to the test system and all residual test substance will be disposed of following finalization of the report.

Deviations

No known deviations from the protocol or test-method SOPs occurred during the conduct of this study.

RESULTS AND DISCUSSION

Solubility Test

Ethanol (EtOH) was selected as the solvent of choice based on solubility of the test substance and compatibility with the target cells. The test substance was a soluble and clear solution in ethanol (EtOH) at approximately 500 mg/mL, the highest concentration tested.

Sterility Results

No contaminant colonies were observed on the sterility plates for the test substance dilutions and the S9 and Sham mixes.

Preliminary Toxicity Test

The results of the preliminary toxicity test are presented in Tables 1 through 5. These data were generated in Experiment A1. In the preliminary toxicity test, the maximum dose tested was 5000 µg per plate; this dose was achieved using a concentration of 100 mg/mL and a 50 µL plating aliquot. The dose levels tested were 6.7, 10, 33, 67, 100, 333, 667, 1000, 3333 and 5000 µg per plate. Toxicity was observed with some test conditions beginning at 3333 or at 5000 µg per plate. Precipitate was observed beginning at 3333 or at 5000 µg per plate. Based on the findings of the preliminary toxicity test, the maximum dose plated in the mutagenicity test was 5000 µg per plate.

Mutagenicity Test

The results of the mutagenicity test are presented in Tables 6 through 15 and summarized in Table 16. These data were generated in Experiment B1. The dose levels tested were 75, 200, 600, 1800 and 5000 µg per plate. Toxicity was observed with some test conditions beginning at 1800 or at 5000 µg per plate. Precipitate was observed at 5000 µg per plate with most test conditions.

In Experiment B1, no positive mutagenic responses were observed with any of the tester strains in either the presence or absence of S9 activation.

Test Substance Characterization (Appendix D)

Non-GLP method development was performed to determine feasibility of characterization of Low DCPD Resin Oil test substance (H-25429). The purpose of this method development was also to adjust the sponsor-provided analytical method to instrumentation being used at DuPont Haskell laboratory. The method provided by the sponsor, as well as the final analytical method used to generate the preliminary feasibility data at DuPont Haskell

laboratory, are in Appendix D. The sponsor also provided the initial lot analysis of the test substance with the associated chromatogram. Both the sponsor's analysis and the chromatogram are in Appendix D. Low DCPD Resin Oil test substance was analyzed by gas chromatography (GC) using flame-ionization detection (FID). The representative chromatogram for the test substance is in Appendix D. Because of the insufficient resolution of p-vinyltoluene, m-vinyltoluene, and 1,2,4-trimethylbenzene in the test substance chromatogram, the sponsor has decided to use the supplier-provided analysis for the test substance characterization.

Based on the relative agreement between the chromatographic profiles of the test substance received at the testing facility (H-25429) and the chromatogram provided by the sponsor, as well as the corresponding % composition values for m-vinyltoluene, p-vinyltoluene, indene, and 1,2,4-trimethylbenzene, we believe that the intended test substance was received at the testing facility.

CONCLUSION

All criteria for a valid study were met as described in the protocol. The results of the Low Dicyclopentadiene Resin Oil: Bacterial Reverse Mutation Test indicate that, under the conditions of this study, Low DCPD Resin Oil did not cause a positive response in either the presence or absence of Aroclor-induced rat liver S9. The study was concluded to be negative without conducting a confirmatory test because no unique metabolism requirements were known about the test substance and because no equivocal responses were observed in the test that would suggest further testing is warranted.

REFERENCES

Ames, B.N., J. McCann and E. Yamasaki (1975) Methods for Detecting Carcinogens and Mutagens with the *Salmonella*/Mammalian Microsome Mutagenicity Test, Mutation Research, 31:347-364.

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Green, M.H.L. and W.J. Muriel (1976) Mutagen testing using trp+ reversion in *Escherichia coli*, Mutation Research 38:3-32.

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OECD Guideline 471 (Genetic Toxicology: Bacterial Reverse Mutation Test), Ninth Addendum to the OECD Guidelines for the Testing of Chemicals, published by OECD, Paris, February 1998.

US EPA Health Effects Test Guidelines, OPPTS Guidelines 870.5100 (1998).

Vogel, H.J. and D.M. Bonner (1956) Acetylornithinase of *E. coli*: Partial Purification and Some Properties, J. Biol. Chem., 218:97-106.

Bacterial Mutation Test
Preliminary Toxicity Test

Table 1

Test Substance Id : Low DCPD Resin Oil
Study Number : AA75HB.502.BTL
Experiment No. : A1
Strain : TA98
Date Plated : 29 May 2003
Vehicle : ethanol (100 %)
Plating Aliquot : 50 µL

Test Substance Concentration µg per plate	With S9 Activation Revertants per plate	With S9 Activation Background Lawn	Without S9 Activation Revertants per plate	Without S9 Activation Background Lawn
Vehicle	15	1	11	1
6.7	11	1	12	1
10	13	1	12	1
33	14	1	13	1
67	14	1	14	1
100	15	1	11	1
333	13	1	10	1
667	15	1	11	1
1000	11	1	10	1
3333	15	1NP	12	2NP
5000	11	2NP	12	2NP

Background Lawn Code
1=Normal; 2=Slightly reduced; 3=Moderately reduced
4=Extremely reduced; 5=Absent; 6=Obscured by precipitate
NP=Non-Interfering precipitate; IP=Interfering precipitate

Bacterial Mutation Test
Preliminary Toxicity Test

Table 2

Test Substance Id : Low DCPD Resin Oil
Study Number : AA75HB.502.BTL
Experiment No. : A1
Strain : TA100
Date Plated : 29 May 2003
Vehicle : ethanol (100 %)
Plating Aliquot : 50 µL

Test Substance Concentration µg per plate	With S9 Activation Revertants per plate	With S9 Activation Background Lawn	Without S9 Activation Revertants per plate	Without S9 Activation Background Lawn
Vehicle	158	1	177	1
6.7	165	1	155	1
10	159	1	152	1
33	152	1	156	1
67	152	1	148	1
100	170	1	150	1
333	182	1	169	1
667	184	1	153	1
1000	179	1	163	1
3333	202	3NP	195	3NP
5000	222	3NP	178	3NP

Background Lawn Code
1=Normal; 2=Slightly reduced; 3=Moderately reduced
4=Extremely reduced; 5=Absent; 6=Obscured by precipitate
NP=Non-Interfering precipitate; IP=Interfering precipitate

Bacterial Mutation Test
Preliminary Toxicity Test

Table 3

Test Substance Id : Low DCPD Resin Oil
Study Number : AA75HB.502.BTL
Experiment No. : A1
Strain : TA1535
Date Plated : 29 May 2003
Vehicle : ethanol (100 %)
Plating Aliquot : 50 µL

Test Substance Concentration µg per plate	With S9 Activation Revertants per plate	With S9 Activation Background Lawn	Without S9 Activation Revertants per plate	Without S9 Activation Background Lawn
Vehicle	10	1	9	1
6.7	11	1	11	1
10	6	1	9	1
33	10	1	8	1
67	8	1	10	1
100	11	1	11	1
333	11	1	11	1
667	10	1	10	1
1000	11	1	7	1
3333	11	1	8	1
5000	7	3NP	9	3NP

Background Lawn Code
1=Normal; 2=Slightly reduced; 3=Moderately reduced
4=Extremely reduced; 5=Absent; 6=Obscured by precipitate
NP=Non-Interfering precipitate; IP=Interfering precipitate

Bacterial Mutation Test
Preliminary Toxicity Test

Table 4

Test Substance Id : Low DCPD Resin Oil
Study Number : AA75HB.502.BTL
Experiment No. : A1
Strain : TA1537
Date Plated : 29 May 2003
Vehicle : ethanol (100 %)
Plating Aliquot : 50 µL

Test Substance Concentration µg per plate	With S9 Activation Revertants per plate	With S9 Activation Background Lawn	Without S9 Activation Revertants per plate	Without S9 Activation Background Lawn
Vehicle	7	1	8	1
6.7	9	1	7	1
10	5	1	4	1
33	4	1	5	1
67	5	1	4	1
100	6	1	6	1
333	8	1	4	1
667	7	1	4	1
1000	6	1	8	1
3333	4	3NP	7	3NP
5000	7	3NP	4	3NP

Background Lawn Code
1=Normal; 2=Slightly reduced; 3=Moderately reduced
4=Extremely reduced; 5=Absent; 6=Obscured by precipitate
NP=Non-Interfering precipitate; IP=Interfering precipitate

Bacterial Mutation Test
Preliminary Toxicity Test

Table 5

Test Substance Id : Low DCPD Resin Oil
Study Number : AA75HB.502.BTL
Experiment No. : A1
Strain : WP2 *uvrA*
Date Plated : 29 May 2003
Vehicle : ethanol (100 %)
Plating Aliquot : 50 µL

Test Substance Concentration µg per plate	With S9 Activation Revertants per plate	With S9 Activation Background Lawn	Without S9 Activation Revertants per plate	Without S9 Activation Background Lawn
Vehicle	10	1	17	1
6.7	12	1	17	1
10	11	1	14	1
33	12	1	10	1
67	11	1	10	1
100	12	1	18	1
333	14	1	15	1
667	11	1	10	1
1000	12	1	12	1
3333	11	1NP	14	1NP
5000	15	1NP	12	1NP

Background Lawn Code
1=Normal; 2=Slightly reduced; 3=Moderately reduced
4=Extremely reduced; 5=Absent; 6=Obscured by precipitate
NP=Non-Interfering precipitate; IP=Interfering precipitate

Bacterial Mutation Test

Table 6

Test Substance Id : Low DCPD Resin Oil
Study Number : AA75HB.502.BTL Experiment No : B1
Strain : TA98 Cells Seeded : 1.0 X 10^8
Liver Microsomes : None Date Plated : 10 Jun 2003
Vehicle : ethanol (100 %)
Plating Aliquot : 50 µL

Concentration µg per plate	Plate Number	Revertants per plate	Background Code	Average Revertants	Standard Deviation
Vehicle	01	13	1		
	02	11	1		
	03	10	1	11	2
75	01	13	1		
	02	10	1		
	03	13	1	12	2
200	01	12	1		
	02	13	1		
	03	13	1	13	1
600	01	11	1		
	02	13	1		
	03	10	1	11	2
1800	01	12	1		
	02	11	1		
	03	9	1	11	2
5000	01	13	1NP		
	02	11	1NP		
	03	12	1NP	12	1
Positive Control 2-nitrofluorene 1.0 µg per plate					
	01	198	1		
	02	156	1		
	03	202	1	185	25

Background Lawn Code
1=Normal; 2=Slightly reduced; 3=Moderately reduced
4=Extremely reduced; 5=Absent; 6=Obscured by precipitate
NP=Non-Interfering precipitate; IP=Interfering precipitate

Bacterial Mutation Test

Table 7

Test Substance Id : Low DCPD Resin Oil
Study Number : AA75HB.502.BTL Experiment No : B1
Strain : TA98 Cells Seeded : 1.0 X 10^8
Liver Microsomes : Rat liver S9 Date Plated : 10 Jun 2003
Vehicle : ethanol (100 %)
Plating Aliquot : 50 µL

Concentration µg per plate	Plate Number	Revertants per plate	Background Code	Average Revertants	Standard Deviation
Vehicle	01	11	1		
	02	14	1		
	03	11	1	12	2
75	01	11	1		
	02	11	1		
	03	15	1	12	2
200	01	12	1		
	02	12	1		
	03	10	1	11	1
600	01	10	1		
	02	12	1		
	03	10	1	11	1
1800	01	11	1		
	02	10	1		
	03	11	1	11	1
5000	01	10	1NP		
	02	11	1NP		
	03	10	1NP	10	1
Positive Control 2-aminoanthracene 1.0 µg per plate					
	01	359	1		
	02	373	1		
	03	327	1	353	24

Background Lawn Code
1=Normal; 2=Slightly reduced; 3=Moderately reduced
4=Extremely reduced; 5=Absent; 6=Obscured by precipitate
NP=Non-Interfering precipitate; IP=Interfering precipitate

Bacterial Mutation Test

Table 8

Test Substance Id : Low DCPD Resin Oil
Study Number : AA75HB.502.BTL Experiment No : B1
Strain : TA100 Cells Seeded : 0.8 X 10^8
Liver Microsomes : None Date Plated : 10 Jun 2003
Vehicle : ethanol (100 %)
Plating Aliquot : 50 µL

Concentration µg per plate	Plate Number	Revertants per plate	Background Code	Average Revertants	Standard Deviation
Vehicle	01	206	1		
	02	175	1		
	03	203	1	195	17
75	01	114	1		
	02	214	1		
	03	196	1	175	53
200	01	179	1		
	02	157	1		
	03	187	1	174	16
600	01	149	2		
	02	176	2		
	03	174	2	166	15
1800	01	276	3		
	02	216	3		
	03	195	3	229	42
5000	01	240	3NP		
	02	217	3NP		
	03	176	3NP	211	32
Positive Control sodium azide 1.0 µg per plate					
	01	600	1		
	02	665	1		
	03	639	1	635	33

Background Lawn Code
1=Normal; 2=Slightly reduced; 3=Moderately reduced
4=Extremely reduced; 5=Absent; 6=Obscured by precipitate
NP=Non-Interfering precipitate; IP=Interfering precipitate

Bacterial Mutation Test

Table 9

Test Substance Id : Low DCPD Resin Oil
Study Number : AA75HB.502.BTL Experiment No : B1
Strain : TA100 Cells Seeded : 0.8 X 10^8
Liver Microsomes : Rat liver S9 Date Plated : 10 Jun 2003
Vehicle : ethanol (100 %)
Plating Aliquot : 50 µL

Concentration µg per plate	Plate Number	Revertants per plate	Background Code	Average Revertants	Standard Deviation
Vehicle	01	170	1		
	02	177	1		
	03	206	1	184	19
75	01	196	1		
	02	230	1		
	03	208	1	211	17
200	01	232	1		
	02	213	1		
	03	228	1	224	10
600	01	234	2		
	02	213	2		
	03	230	2	226	11
1800	01	213	2		
	02	235	2		
	03	277	2	242	33
5000	01	219	3NP		
	02	220	3NP		
	03	206	3NP	215	8
Positive Control 2-aminoanthracene 1.0 µg per plate					
	01	622	1		
	02	635	1		
	03	645	1	634	12

Background Lawn Code
1=Normal; 2=Slightly reduced; 3=Moderately reduced
4=Extremely reduced; 5=Absent; 6=Obscured by precipitate
NP=Non-Interfering precipitate; IP=Interfering precipitate

Bacterial Mutation Test

Table 10

Test Substance Id : Low DCPD Resin Oil
Study Number : AA75HB.502.BTL Experiment No : B1
Strain : TA1535 Cells Seeded : 0.9 X 10^8
Liver Microsomes : None Date Plated : 10 Jun 2003
Vehicle : ethanol (100 %)
Plating Aliquot : 50 µL

Concentration µg per plate	Plate Number	Revertants per plate	Background Code	Average Revertants	Standard Deviation
Vehicle	01	12	1		
	02	7	1		
	03	7	1	9	3
75	01	7	1		
	02	11	1		
	03	6	1	8	3
200	01	12	1		
	02	12	1		
	03	11	1	12	1
600	01	12	2		
	02	5	2		
	03	11	2	9	4
1800	01	6	2		
	02	13	2		
	03	5	2	8	4
5000	01	8	3NP		
	02	7	3NP		
	03	9	3NP	8	1
Positive Control sodium azide 1.0 µg per plate					
	01	334	1		
	02	337	1		
	03	371	1	347	21

Background Lawn Code
1=Normal; 2=Slightly reduced; 3=Moderately reduced
4=Extremely reduced; 5=Absent; 6=Obscured by precipitate
NP=Non-Interfering precipitate; IP=Interfering precipitate

Bacterial Mutation Test

Table 11

Test Substance Id : Low DCPD Resin Oil
Study Number : AA75HB.502.BTL Experiment No : B1
Strain : TA1535 Cells Seeded : 0.9 X 10^8
Liver Microsomes : Rat liver S9 Date Plated : 10 Jun 2003
Vehicle : ethanol (100 %)
Plating Aliquot : 50 µL

Concentration µg per plate	Plate Number	Revertants per plate	Background Code	Average Revertants	Standard Deviation
Vehicle	01	9	1		
	02	9	1		
	03	12	1	10	2
75	01	9	1		
	02	4	1		
	03	13	1	9	5
200	01	13	1		
	02	12	1		
	03	8	1	11	3
600	01	15	1		
	02	12	1		
	03	15	1	14	2
1800	01	7	2		
	02	11	2		
	03	12	2	10	3
5000	01	12	3NP		
	02	8	3NP		
	03	8	3NP	9	2
Positive Control 2-aminoanthracene 1.0 µg per plate					
	01	138	1		
	02	87	1		
	03	122	1	116	26

Background Lawn Code
1=Normal; 2=Slightly reduced; 3=Moderately reduced
4=Extremely reduced; 5=Absent; 6=Obscured by precipitate
NP=Non-Interfering precipitate; IP=Interfering precipitate

Bacterial Mutation Test

Table 12

Test Substance Id : Low DCPD Resin Oil
Study Number : AA75HB.502.BTL Experiment No : B1
Strain : TA1537 Cells Seeded : 0.4 X 10^8
Liver Microsomes : None Date Plated : 10 Jun 2003
Vehicle : ethanol (100 %)
Plating Aliquot : 50 µL

Concentration µg per plate	Plate Number	Revertants per plate	Background Code	Average Revertants	Standard Deviation
Vehicle	01	7	1		
	02	6	1		
	03	6	1	6	1
75	01	3	1		
	02	5	1		
	03	5	1	4	1
200	01	5	1		
	02	4	1		
	03	4	1	4	1
600	01	5	2		
	02	5	2		
	03	4	2	5	1
1800	01	3	3		
	02	4	3		
	03	5	3	4	1
5000	01	4	3NP		
	02	3	3NP		
	03	4	3NP	4	1
Positive Control 9-aminoacridine 75 µg per plate					
	01	1158	1		
	02	1126	1		
	03	1125	1	1136	19

Background Lawn Code
1=Normal; 2=Slightly reduced; 3=Moderately reduced
4=Extremely reduced; 5=Absent; 6=Obscured by precipitate
NP=Non-Interfering precipitate; IP=Interfering precipitate

Bacterial Mutation Test

Table 13

Test Substance Id : Low DCPD Resin Oil
Study Number : AA75HB.502.BTL Experiment No : B1
Strain : TA1537 Cells Seeded : 0.4 X 10^8
Liver Microsomes : Rat liver S9 Date Plated : 10 Jun 2003
Vehicle : ethanol (100 %)
Plating Aliquot : 50 µL

Concentration µg per plate	Plate Number	Revertants per plate	Background Code	Average Revertants	Standard Deviation
Vehicle	01	3	1		
	02	6	1		
	03	6	1	5	2
75	01	4	1		
	02	7	1		
	03	6	1	6	2
200	01	3	1		
	02	5	1		
	03	4	1	4	1
600	01	5	1		
	02	5	1		
	03	5	1	5	0
1800	01	5	3		
	02	5	3		
	03	4	3	5	1
5000	01	5	3		
	02	3	3		
	03	4	3	4	1
Positive Control 2-aminoanthracene 1.0 µg per plate					
	01	58	1		
	02	86	1		
	03	64	1	69	15

Background Lawn Code
1=Normal; 2=Slightly reduced; 3=Moderately reduced
4=Extremely reduced; 5=Absent; 6=Obscured by precipitate
NP=Non-Interfering precipitate; IP=Interfering precipitate

Bacterial Mutation Test

Table 14

Test Substance Id : Low DCPD Resin Oil
Study Number : AA75HB.502.BTL Experiment No : B1
Strain : WP2 *uvrA* Cells Seeded : 2.8 X 10^8
Liver Microsomes : None Date Plated : 10 Jun 2003
Vehicle : ethanol (100 %)
Plating Aliquot : 50 µL

Concentration µg per plate	Plate Number	Revertants per plate	Background Code	Average Revertants	Standard Deviation
Vehicle	01	10	1		
	02	11	1		
	03	10	1	10	1
75	01	11	1		
	02	10	1		
	03	10	1	10	1
200	01	10	1		
	02	11	1		
	03	10	1	10	1
600	01	11	1		
	02	11	1		
	03	10	1	11	1
1800	01	11	1		
	02	10	1		
	03	11	1	11	1
5000	01	12	2NP		
	02	11	2NP		
	03	10	2NP	11	1
Positive Control methyl methanesulfonate 1000 µg per plate					
	01	115	1		
	02	92	1		
	03	104	1	104	12

Background Lawn Code
1=Normal; 2=Slightly reduced; 3=Moderately reduced
4=Extremely reduced; 5=Absent; 6=Obscured by precipitate
NP=Non-Interfering precipitate; IP=Interfering precipitate

Bacterial Mutation Test

Table 15

Test Substance Id : Low DCPD Resin Oil
Study Number : AA75HB.502.BTL Experiment No : B1
Strain : WP2 *uvr*A Cells Seeded : 2.8 X 10^8
Liver Microsomes : Rat liver S9 Date Plated : 10 Jun 2003
Vehicle : ethanol (100 %)
Plating Aliquot : 50 µL

Concentration µg per plate	Plate Number	Revertants per plate	Background Code	Average Revertants	Standard Deviation
Vehicle	01	10	1		
	02	11	1		
	03	13	1	11	2
75	01	12	1		
	02	12	1		
	03	11	1	12	1
200	01	11	1		
	02	12	1		
	03	10	1	11	1
600	01	11	1		
	02	12	1		
	03	13	1	12	1
1800	01	10	1		
	02	11	1		
	03	13	1	11	2
5000	01	13	1NP		
	02	8	1NP		
	03	10	1NP	10	3
Positive Control 2-aminoanthracene 10 µg per plate					
	01	373	1		
	02	427	1		
	03	482	1	427	55

Background Lawn Code
1=Normal; 2=Slightly reduced; 3=Moderately reduced
4=Extremely reduced; 5=Absent; 6=Obscured by precipitate
NP=Non-Interfering precipitate; IP=Interfering precipitate

Bacterial Mutation Test
Summary of Results

Table 16

Test Substance Id : Low DCPD Resin Oil
Study Number : AA75HB.502.BTL Experiment No : B1

Average Revertants Per Plate ± Standard Deviation

Liver Microsomes: None

Dose (µg/plate)	TA98	TA100	TA1535	TA1537	WP2 *uvr*A
Vehicle	11 ± 2	195 ± 17	9 ± 3	6 ± 1	10 ± 1
75	12 ± 2	175 ± 53	8 ± 3	4 ± 1	10 ± 1
200	13 ± 1	174 ± 16	12 ± 1	4 ± 1	10 ± 1
600	11 ± 2	166 ± 15	9 ± 4	5 ± 1	11 ± 1
1800	11 ± 2	229 ± 42	8 ± 4	4 ± 1	11 ± 1
5000	12 ± 1	211 ± 32	8 ± 1	4 ± 1	11 ± 1
Positive	185 ± 25	635 ± 33	347 ± 21	1136 ± 19	104 ± 12

Liver Microsomes: Rat liver S9

Dose (µg/plate)	TA98	TA100	TA1535	TA1537	WP2 *uvr*A
Vehicle	12 ± 2	184 ± 19	10 ± 2	5 ± 2	11 ± 2
75	12 ± 2	211 ± 17	9 ± 5	6 ± 2	12 ± 1
200	11 ± 1	224 ± 10	11 ± 3	4 ± 1	11 ± 1
600	11 ± 1	226 ± 11	14 ± 2	5 ± 0	12 ± 1
1800	11 ± 1	242 ± 33	10 ± 3	5 ± 1	11 ± 2
5000	10 ± 1	215 ± 8	9 ± 2	4 ± 1	10 ± 3
Positive	353 ± 24	634 ± 12	116 ± 26	69 ± 15	427 ± 55

Vehicle = Vehicle Control
Positive = Positive Control
Plating aliquot: 50 µL

APPENDIX A

Historical Control Data

Historical Negative and Positive Control Values
2000 – 2002

revertants per plate

Strain	Control	Activation							
		None				Rat Liver			
		Mean	SD	Min	Max	Mean	SD	Min	Max
TA98	Neg	16	6	5	59	21	7	5	58
	Pos	239	158	30	1581	679	383	40	2294
TA100	Neg	148	36	68	262	152	38	74	271
	Pos	572	149	240	1945	904	437	163	2922
TA1535	Neg	15	6	3	46	13	5	2	50
	Pos	319	131	16	978	137	80	11	2246
TA1537	Neg	7	3	1	22	8	3	1	28
	Pos	634	375	13	2389	127	133	12	2060
WP2 *uvr*A	Neg	14	4	5	52	14	4	4	52
	Pos	164	147	14	1809	390	255	22	1493

SD=standard deviation; Min=minimum value; Max=maximum value; Neg=negative control (including but not limited to deionized water, dimethyl sulfoxide, ethanol and acetone); Pos=positive control

These values are calculated based on the individual plate counts and each value is based on more than 1000 observations.

APPENDIX B

Study Protocol

Received by RAFOA 28 May 2003

Sponsor Project Number: DuPont-12984

BioReliance Study Number: AA75HB.502.BTL

Low Dicyclopentadiene Resin Oil: Bacterial Reverse Mutation Test

Work Request Number 14295

QA ES 5/29/03
APPROVED

Service Code 500

Protocol

American Chemistry Council Reference Number: OLF-92.0-HPV789-DHL

Sponsor Project Number: DuPont-12984

BioReliance Study Number: AA75HB.502.BTL

Table of Contents

Sponsor Project Number: DuPont-12984

BioReliance Study Number: AA75HB.502.BTL

Low Dicyclopentadiene Resin Oil: Bacterial Reverse Mutation Test

1.0 PURPOSE

The purpose of this study is to evaluate the mutagenic potential of the test substance by measuring its ability to induce reverse mutations at selected loci of several strains of *Salmonella typhimurium* and at the tryptophan locus of *Escherichia coli* WP2 *uvr*A in the presence and absence of S9 activation.

2.0 SPONSOR

2.1	Sponsor Name:	American Chemistry Council Olefins Panel
2.2	Study Monitor:	Maria Donner, Ph.D. E.I. du Pont de Nemours and Company DuPont Haskell Laboratory P.O. Box 50, 1090 Elkton Road Newark, DE 19714-0050 Phone: 302-366-5251 Fax: 302-366-5207 Email: maria.donner@usa.dupont.com
2.3	Sponsor Project No.:	DuPont-12984
2.4	WR#:	14295
2.5	Haskell #:	H-25429
2.6	Service Code:	500

3.0 IDENTIFICATION OF TEST AND CONTROL SUBSTANCES

3.1	Test Substance Name:		Low Dicyclopentadiene Resin Oil
3.2	Test Substance CAS No.:		68477-54-3
3.3	Test Substance Lot No.:		Not applicable
3.4	Test Substance I.D.:		Low DCPD Resin oil (to be used in report text)
3.5	Controls:	Negative:	Test substance vehicle
		Positive:	9-aminoacridine, CAS No. 90-45-9 2-aminoanthracene, CAS No. 613-13-8 methyl methanesulfonate, CAS No. 66-27-3

Sponsor Project Number: DuPont-12984

BioReliance Study Number: AA75HB.502.BTL

2-nitrofluorene, CAS No. 607-57-8
sodium azide, CAS No. 26628-22-8

3.6 Test Substance Storage and Handling

The test substance will be supplied as a liquid and stored refrigerated and protected from light and air. The original container will be kept blanketed with nitrogen. Material to be used on the study may be dispensed into a clear glass container and does not need to be handled in any special manner.

3.7 Test Substance Characterization

Unless alternate arrangements are made, the testing facility at BioReliance will not perform analysis of the dosing solutions. The Sponsor will be directly responsible for determination and documentation of the analytical purity and composition of the test substance, and the stability and strength of the test substance in the solvent (or vehicle). The sponsor has not determined a purity value (% active ingredient) for the Low Dicyclopentadiene Resin Oil. A correction factor will not be used for preparation of the dosing solutions.

The test substance will be characterized by Haskell Laboratory prior to the preliminary toxicity test. Stability of the test substance will be established by Haskell Laboratory. The analytical methods used will be documented in the Haskell Analytical Chemistry Group study records.

3.8 Test Substance Retention Sample

The retention of a reserve sample of the test substance will be the responsibility of the Sponsor.

4.0 TESTING FACILITY AND KEY PERSONNEL

4.1	Name:	Toxicology Testing Facility BioReliance
4.2	Address:	9630 Medical Center Drive Rockville, MD 20850
4.3	Study Director:	Valentine O. Wagner III, M.S. Phone: 301-610-2152 Fax: 301-738-2362 Email: swagner@bioreliance.com

Sponsor Project Number: DuPont-12984

BioReliance Study Number: AA75HB.502.BTL

5.0 PROPOSED STUDY DATES

5.1	Experimental Start Date:	May 30, 2003
5.2	Experimental Termination Date:	July 03, 2003
5.3	Draft Report Date:	July 17, 2003
5.4	Final Report Date:	2 weeks after Study Monitor approves draft

6.0 TEST SYSTEM

The tester strains will include the *S. typhimurium* histidine auxotrophs TA98, TA100, TA1535 and TA1537 as described by Ames *et al.* (1975) and the *E. coli* tester strain WP2 *uvr*A as described by Green and Muriel (1976).

Histidine Mutation			Tryptophan Mutation	Additional Mutations		
*his*G46	*his*C3076	*his*D3052	*Trp*E	LPS	Repair	R-factor
TA1535	TA1537	-	-	rfa	Δ*uvr*B	-
TA100	-	TA98	-	rfa	Δ*uvr*B	+R
-	-	-	WP2 *uvr*A	-	Δ*uvr*A	-

Each *S. typhimurium* tester strain contains, in addition to a mutation in the histidine operon, additional mutations that enhance sensitivity to some mutagens. The *rfa* mutation results in a cell wall deficiency that increases the permeability of the cell to certain classes of chemicals such as those containing large ring systems that would otherwise be excluded. The deletion in the *uvr*B gene results in a deficient DNA excision-repair system. Tester strains TA98 and TA100 also contain the pKM101 plasmid (carrying the R-factor). It has been suggested that the plasmid increases sensitivity to mutagens by modifying an existing bacterial DNA repair polymerase complex involved with the mismatch-repair process.

TA98 and TA1537 are reverted from histidine dependence (auxotrophy) to histidine independence (prototrophy) by frameshift mutagens. TA100 is reverted by both frameshift and base substitution mutagens and TA1535 is reverted only by mutagens that cause base substitutions.

The *E. coli* tester strain has an AT base pair at the critical mutation site within the *trp*E gene (Wilcox *et al.*, 1990). Tester strain WP2 *uvr*A has a deletion in the *uvr*A gene resulting in a deficient DNA excision-repair system. Tryptophan revertants can arise due to a base change at the originally mutated site or by a base change elsewhere in the chromosome causing the original mutation to be suppressed. Thus, the specificity of the reversion mechanism is sensitive to base-pair substitution mutations (Green and Muriel, 1976).

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The *S. typhimurium* tester strains were received directly from Dr. Bruce Ames, University of California, Berkeley or a vendor authorized by his laboratory. The *E. coli* tester strain was received from the National Collection of Industrial and Marine Bacteria, Aberdeen, Scotland (United Kingdom).

7.0 EXPERIMENTAL DESIGN AND METHODOLOGY

The test substance will be tested at a minimum of five dose levels along with appropriate negative and positive controls with tester strains TA98, TA100, TA1535, TA1537 and WP2 *uvr*A with and without S9 activation. All dose levels of test substance, negative controls and positive controls will be plated in triplicate.

7.1 Solubility Determination

Unless the Sponsor has indicated the test substance vehicle, a solubility determination will be conducted to determine the maximum soluble concentration or workable suspension up to a maximum of 50 mg/mL for aqueous vehicles and 500 mg/mL for organic vehicles. Vehicles compatible with this test system, in order of preference, include but are not limited to deionized water (CAS 7732-18-5), dimethyl sulfoxide (CAS 67-68-5), ethanol (CAS 64-17-5) and acetone (CAS 67-64-1). The vehicle of choice will be the solvent, selected in order of preference, which permits preparation of the highest workable/soluble stock concentration, up to 50 mg/mL for aqueous vehicles and 500 mg/mL for organic vehicles.

7.2 Preliminary Toxicity Test to Select Dose Levels

Selection of dose levels for the mutagenicity assay will be based upon the toxicity and precipitation profile of the test substance assessed in a preliminary toxicity assay. This preliminary assay will be conducted by exposing TA98, TA100, TA1535, TA1537 and WP2 *uvr*A to negative controls and to at least eight concentrations of test substance, one plate per dose level, in both the presence and absence of S9 activation. Unless indicated otherwise by the Sponsor, the highest dose will be the highest workable concentration in the vehicle of choice but not to exceed 5 mg/plate. In selecting dose levels for the mutagenicity assay the following guidelines will be employed. Doses will be selected such that precipitate does not interfere with manual scoring. Whenever possible, the highest dose for the mutagenicity assay will be selected to give some indication of toxicity without exceeding 5 mg/plate. For freely soluble, nontoxic test substances, the highest dose level will be 5 mg/plate. For precipitating, nontoxic test substances, the highest dose level will be selected in an attempt to yield precipitate at only the top one or two dose levels. The Sponsor will be consulted regarding dose selection if (1) the maximum dose level is selected based on precipitation and this dose level is less than 5 mg/plate or (2) the maximum achievable test substance dose level is less than 5 mg/plate and this dose level is nontoxic.

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7.3 Frequency and Route of Administration

The test system will be exposed to the test substance via the plate incorporation methodology originally described by Ames *et al.* (1975) and updated by Maron and Ames (1983). This test system has been shown to detect a wide range of classes of chemical mutagens (McCann *et al.*, 1975; McCann and Ames, 1976).

If the Sponsor is aware of specific metabolic requirements (e.g., azo compounds), this information will be utilized in designing the assay. Verification of a clear positive response is not required. Negative results will not be retested when justification can be provided. Equivocal results will be retested in consultation with the Sponsor using an appropriate modification of the experimental design (e.g., dose levels, activation system or treatment method). This guidance is based on the OECD Guideline 471 (adopted July 1997 and published February 1998) and ICH Guidance on Specific Aspects of Regulatory Genotoxicity Tests for Pharmaceuticals (1997).

7.4 Controls

7.4.1 Positive Controls

All combinations of positive controls and tester strains plated concurrently with the assay are listed below. All positive controls will be diluted in dimethyl sulfoxide (DMSO) except for sodium azide, which will be diluted in water.

Strain	S9 Activation	Positive Control	Concentration (µg/plate)
Salmonella Strains	Rat	2-aminoanthracene	1.0
WP2 *uvr*A			10
TA98	None	2-nitrofluorene	1.0
TA100, TA1535		sodium azide	1.0
TA1537		9-aminoacridine	75
WP2 *uvr*A		methyl methanesulfonate	1,000

7.4.2 Negative Controls

Appropriate negative controls will be plated for each tester strain with and without S9 activation. The negative control will be the vehicle alone, unless there is no historical basis for use of the selected vehicle. In the latter case, both untreated and vehicle controls will be used.

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7.4.3 Sterility Controls

The most concentrated test substance dilution and the Sham and S9 mixes will be checked for sterility.

7.5 Exogenous Metabolic Activation

Aroclor 1254-induced rat liver S9 will be used as the metabolic activation system. The S9 homogenate will be prepared from male Sprague-Dawley rats induced with a single intraperitoneal injection of Aroclor 1254, 500 mg/kg, five days prior to sacrifice. The S9 will be batch prepared and stored frozen at approximately -70°C until used. Each batch of S9 homogenate will be assayed for its ability to metabolize 2-aminoanthracene and 7,12-dimethylbenzanthracene to forms mutagenic to *S. typhimurium* TA100.

Immediately prior to use, the S9 will be thawed and mixed with a cofactor pool to contain 10% S9 homogenate, 5 mM glucose-6-phosphate, 4 mM β-nicotinamide-adenine dinucleotide phosphate, 8 mM $MgCl_2$ and 33 mM KCl in a 100 mM phosphate buffer at pH 7.4. This mixture is referred to as S9 mix. Sham mix will be 100 mM phosphate buffer at pH 7.4.

7.6 Preparation of Tester Strain

Overnight cultures will be prepared by inoculating from the appropriate master plate (stored at 2 to 8°C) or from the appropriate frozen permanent stock (stored at –70°C or colder) into a vessel containing ~50 mL of culture medium. To assure that cultures are harvested in late log phase, the length of incubation will be controlled and monitored by spectrophotometric monitoring of culture turbidity. Following inoculation, each flask will be placed in a resting shaker/incubator at room temperature. The shaker/incubator will be programmed to begin shaking at approximately 125 rpm at 37±2°C approximately 12 hours before the anticipated time of harvest. Each culture will be harvested at a percent transmittance yielding a titer of approximately 10^9 cells per milliliter. The actual titers will be determined by viable count assays on nutrient agar plates.

7.7 Test System Identification

Each plate will be labeled with a code system that identifies the test substance, test phase, dose level, tester strain and activation type as described in BioReliance's Standard Operating Procedures.

7.8 Test Substance Preparation

Unless specified otherwise, test substance dilutions will be prepared immediately prior to use. All test substance dosing will be at room temperature under yellow light.

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7.9 Treatment of Test System

One half milliliter (0.5 mL) of S9 mix or Sham mix, 100 µL of tester strain and 50 µL of vehicle, test substance dilution or positive control will be added to 2.0 mL of molten selective top agar at 45±2°C. When necessary to achieve the target concentration or eliminate toxic vehicle effects, aliquots of other than 50 µL of test substance/vehicle/positive control will be plated. When plating untreated controls, the addition of test article, vehicle and positive control will be omitted. The mixture will be vortex mixed and overlaid onto the surface of 25 mL of minimal bottom agar. After the overlay has solidified, the plates will be inverted and incubated for approximately 48 to 72 hours at 37±2°C. Plates that are not counted immediately following the incubation period will be stored at 2-8°C.

7.10 Scoring

The condition of the bacterial background lawn will be evaluated for evidence of test substance toxicity and precipitate. Evidence of toxicity will be scored relative to the negative control plate and recorded along with the revertant count for that plate. Toxicity will be evaluated as a decrease in the number of revertant colonies per plate and/or a thinning or disappearance of the bacterial background lawn. Precipitation will be evaluated after the incubation period by visual examination without magnification. Revertant colonies for a given tester strain and activation condition, except for positive controls, will be counted either entirely by automated colony counter or entirely by hand unless the plate exhibits toxicity.

7.11 Tester Strain Verification

On the day of use in the mutagenicity assay, all tester strain cultures will be checked for the appropriate genetic markers cited in §6.0.

8.0 CRITERIA FOR DETERMINATION OF A VALID TEST

The following criteria must be met for the mutagenicity assay to be considered valid:

8.1 Tester Strain Integrity

To demonstrate the presence of the *rfa* mutation, all *S. typhimurium* tester strain cultures must exhibit sensitivity to crystal violet. To demonstrate the presence of the *uvr*B mutation, all *S. typhimurium* tester strain cultures must exhibit sensitivity to ultraviolet light. To demonstrate the presence of the *uvr*A mutation, all *E. coli* tester strain cultures must exhibit sensitivity to ultraviolet light. To demonstrate the presence of the pKM101 plasmid R-factor, tester strain cultures of TA98 and TA100 must exhibit resistance to ampicillin.

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8.2 Negative Control Values

Based on historical control data, all tester strain cultures must exhibit characteristic number of spontaneous revertants per plate in the negative controls (vehicle). The mean revertants per plate must be within the following ranges (inclusive): TA98, 10 - 50; TA100, 80 - 240; TA1535, 5 - 45; TA1537, 3 - 21; WP2 *uvr*A, 10 - 60. Untreated controls, when part of the design, must also be within the ranges cited above.

8.3 Tester Strain Titers

To ensure that appropriate numbers of bacteria are plated, all tester strain culture titers must be equal to or greater than 0.3×10^9 cells per milliliter.

8.4 Positive Control Values

Each mean positive control value must exhibit at least a 3.0-fold increase over the respective mean negative control value (vehicle) for each tester strain. The positive control value for each strain and activation condition must also be within historical minimum and maximum control values.

8.5 Toxicity

A minimum of three non-toxic dose levels will be required to evaluate assay data. A dose level is considered toxic if it causes a >50% reduction in the mean number of revertants per plate relative to the mean negative control value (this reduction must be accompanied by an abrupt dose-dependent drop in the revertant count) or a reduction in the background lawn. In the event that less than three non-toxic dose levels are achieved, the affected portion of the assay will be repeated with an appropriate change in dose levels.

9.0 EVALUATION OF TEST RESULTS

For a test substance to be evaluated positive, it must cause a dose-related increase in the mean revertants per plate of at least one tester strain over a minimum of two increasing concentrations of test substance as specified below:

9.1 Strains TA1535 and TA1537

Data sets will be judged positive if the increase in mean revertants at the peak of the dose response is equal to or greater than 3.0-times the mean negative control value (vehicle).

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9.2 Strains TA98, TA100 and WP2 *uvr*A

Data sets will be judged positive if the increase in mean revertants at the peak of the dose response is equal to or greater than 2.0-times the mean negative control value (vehicle).

Responses that do not meet the above criteria for evaluation as positive will be evaluated as negative. Equivocal responses will be evaluated based on the professional judgement of the Study Director on a case-by-case basis.

10.0 REPORT

A report of the results of this study will be prepared by the Testing Laboratory and will accurately describe all methods used for generation and analysis of the data. The report will include:

- Test Substance: identification and CAS no., if known; physical nature and purity, if known; physicochemical properties relevant to the conduct of the study, if known; stability of test substance, if known.
- Solvent/Vehicle: justification for choice of vehicle; solubility and stability of test substance in solvent/vehicle, if known.
- Strains: strains used; number of cells/mL per culture; strain characteristics.
- Test conditions: amount of test substance per plate with rationale for dose selection and number of plates per concentration; media used; type and composition of metabolic activation system, including acceptability criteria; treatment procedures.
- Results: signs of toxicity; signs of precipitation; individual plate counts; the mean number of revertant colonies per plate and standard deviation; dose-response relationship, where possible; statistical analysis, if any; concurrent negative and positive control data means and standard deviations; historical negative and positive control data with ranges, means and standard deviation.
- Discussion of results
- Conclusion
- Statement of Compliance
- Quality Assurance Statement
- A robust summary, provided by the Study Monitor and reviewed by the Study Director, will be included in the final report.

11.0 RECORDS AND ARCHIVES

At the completion of the study, all raw data, the protocol and all reports for procedures performed at BioReliance will be archived at E.I. du Pont de Nemours and Company per the contractual arrangements between the American Chemistry Council and E.I. du Pont

de Nemours and Company. All study materials returned to the E.I. du Pont de Nemours and Company will first be copied and the copy will be retained in the BioReliance archives for a minimum of 10 years.

12.0 REGULATORY REQUIREMENTS/GOOD LABORATORY PRACTICE

This protocol has been written to comply with OECD Guideline 471 (Genetic Toxicology: Bacterial Reverse Mutation Assay), Ninth Addendum to the OECD Guidelines for the Testing of Chemicals, published by OECD, Paris, February 1998, with OPPTS Guideline 870.5100 (Bacterial Reverse Mutation Test) and with EC Commission Directive 2000/32/EC.

Portions of this study conducted at BioReliance will be performed in compliance with the provisions of the U.S. EPA Good Laboratory Practice Regulations (40 CFR 792). The protocol, an in-process phase, the raw data, and report(s) will be audited per the Standard Operating Procedures (SOPs) of BioReliance by the Quality Assurance Unit of BioReliance for compliance with GLPs, the SOPs of BioReliance and the study protocol. At least one, study-specific, in-process inspection will be performed for this study. A signed QA statement will be included in the final report. This statement will list the study-specific phases inspected, the dates of each inspection, and the dates the results of each inspection were reported to the Study Director and the Study Director's management. In addition, a signed GLP compliance statement will be included in the final report. This statement will cite the GLP guideline(s) with which the study is compliant and any exceptions to this compliance, if applicable, including the omission of characterization or stability analyses of the test or control articles or their mixtures.

Raw data, the protocol and reports generated at facilities other than BioReliance will or will not be QA audited per the contractual arrangements between that facility and the Sponsor.

Unless arrangements are made to the contrary, unused dosing solutions will be disposed of following administration to the test system and all residual test substance will be disposed of following finalization of the report.

13.0 REFERENCES

Ames, B.N., McCann, J. and Yamasaki, E. (1975). Methods for detecting carcinogens and mutagens with the *Salmonella*/mammalian-microsome mutagenicity test. Mutation Research 31:347-364.

EC Commission Directive 2000/30/EC.

Green, M.H.L., and Muriel, W.J. (1976). Mutagen testing using trp^+ reversion in *Escherichia coli*. Mutation Research 38:3-32.

Sponsor Project Number: DuPont-12984

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Regulatory Genotoxicity Tests for Pharmaceuticals. S2A document recommended for adoption at step 4 of the ICH process on July 19, 1995. Federal Register 61:18198-18202, April 24, 1996.

International Conference on Harmonisation (ICH) of Technical Requirements for Registration of Pharmaceuticals for Human Use. Genotoxicity: A Standard Battery for Genotoxicity Testing of Pharmaceuticals. S2B document recommended for adoption at step 4 of the ICH process on July 16, 1997. Federal Register 62:16026-16030, November 21, 1997.

McCann, J. and Ames, B.N. (1976). Detection of carcinogens as mutagens in the *Salmonella*/microsome test: assay of 300 chemicals: discussion. Proc. Natl. Acad. Sci. USA 73:950-954.

McCann, J., Choi, E., Yamasaki, E. and Ames, B.N. (1975). Detection of carcinogens as mutagens in the *Salmonella*/microsome test: assay of 300 chemicals. Proc. Natl. Acad. Sci. USA 72:5135-5139.

Maron, D.M. and Ames, B.N. (1983). Revised Methods for the *Salmonella* Mutagenicity Test. Mutation Research 113:173-215.

OECD Guideline 471 (Genetic Toxicology: Bacterial Reverse Mutation Test), Ninth Addendum to the OECD Guidelines for the Testing of Chemicals, published by OECD, Paris, February 1998.

Wilcox, P., Naidoo, A., Wedd, D.J. and Gatehouse, D.G. (1990). Comparison of *Salmonella typhimurium* TA102 with *Escherichia coli* WP2 tester strains. Mutagenesis 5:285-291.

14.0 APPROVAL

Maria Donner — 23-May-2003
Maria Donner, Ph.D. — Date
Study Monitor

Valentine O. Wagner, III — 27 May 2003
BioReliance Study Director — Date

[signature] — 28 May 2003
BioReliance Study Management — Date

APPENDIX C

Robust Summary

Robust Summary: Category
Genetic Toxicity - in Vitro

Test Substance *Test substance*	Low Dicyclopentadiene (DCPD) Resin Oil, CAS# 68477-54-3; stable at room temperature below 70° F; colorless- light yellow liquid Olefins Panel HPV Stream Name: Low DCPD Resin Oil Low DCPD Resin Oil is a C8 to C10 distillate obtained from a pyrolysis gasoline stream produced by an ethylene production process (steam cracking process). The sample tested consisted of vinyltoluenes (17%), trimethylbenzenes (9%), styrene and methylstyrenes (3%), indene (14%), methylindene (8%), and naphthalene (1%). The balance of the composition is expected to consist of other hydrocarbons with carbon range primarily 8 to 10, mainly aromatics and olefins, with some paraffins. Note: the above composition percentages were summarized from data reported by the supplier of the test substance on February 10, 2003.
Method Methods/guidelines followed	OECD Guideline 471 (Genetic Toxicology: Bacterial Reverse Mutation Test), adopted July 1997 (published February 1998), OPPTS Guideline 870.5100 (Bacterial Reverse Mutation Test) and EC Commission Directive 2000/32/EC.
System of testing	*Salmonella typhimurium* and *Escherichia coli* with and without S9
GLP	Yes
Year	2003
Species/Strain	*S. typhimurium* TA98, TA100, TA1535, TA1537 and *E. coli* WP2 *uvr*A.
Metabolic activation	Yes
Species and cell type Quantity Induced or not induced	Sprague-Dawley rat liver (S9 fraction) prepared in-house 10% S9 in S9 mix Aroclor 1254 induced, rats were given 500mg/kg ip 5 days prior to sacrifice
Concentrations tested	75, 200, 600, 1800 and 5000 µg/plate
Statistical Methods	None
Remarks for Test Conditions	Criteria for positive response were a dose-related increase in the mean revertants per plate of at least one tester strain over a minimum of two increasing concentrations as specified below: TA1535, TA1537: At the peak of the dose response an equal to or greater than 3.0-fold dose related increase over solvent control values with or without metabolic activation. TA98, TA100, *E. coli* WP2 *uvr*A: At the peak of the dose response an

	equal to or greater than 2.0-fold dose related increase over solvent control values with or without metabolic activation. Negative controls: Based on historical control data, all tester strains must exhibit characteristic numbers of spontaneous revertants per plate. Positive controls: The mean of each positive control value must exhibit at least a 3.0-fold increase over the respective mean negative control value (vehicle) for each tester strain. Low DCPD Resin Oil test solutions were prepared in ethanol immediately prior to use. *Salmonella* strains and *E. coli* WP2 *uvr*A (approx. 10^9 cells/mL) were exposed to either test solution or vehicle ±S9 by the plate incorporation method. The preliminary toxicity test was conducted prior to the mutagenicity test with all tester strains over a range of 6.7 to 5000 µg/plate (one plate per dose) ±S9. The dose levels tested in the mutagenicity test were 75, 200, 600, 1800 and 5000 µg/plate ±S9. The mutagenicity test was conducted on triplicate plates per dose. Five hundred (500) microliters of S9 or Sham mix, 100 µL of tester strain and 50 µL vehicle or test substance dilution were added to 2.0 mL of molten selective top agar at 45±2°C. After vortexing, the mixture was overlaid onto the surface of minimal agar plates. After the overlay had solidified, the plates were inverted and incubated for approximately 48 to 72 hours at 37±2°C. Revertant colonies for a given tester strain and activation condition, except for the positive controls, were counted either entirely by automated colony counter or entirely by hand unless the plate exhibited toxicity, and conditions of background lawn and precipitation were evaluated. Positive control compounds for the –S9 condition were: 2-nitrofluorene (1.0 µg/plate) for TA98; sodium azide (1.0 µg/plate) for TA100 and TA1535; 9-aminoacridine (75 µg/plate) for TA1537; and methyl methanesulfonate (1000 µg/plate) for WP2*uvr*A. The positive control compound for the +S9 condition was 2-aminoanthracene, 1.0 µg/plate for all *Salmonella* strains, and 10 µg/plate for WP2*uvr*A.
Results Genotoxic effects	In the preliminary toxicity test, the maximum dose tested was 5000 µg per plate; this dose was achieved using a concentration of 100 mg/mL and a 50 µL plating aliquot. The dose levels tested were 6.7, 10, 33, 67, 100, 333, 667, 1000, 3333 and 5000 µg per plate. Toxicity was observed with some conditions beginning at 3333 or at 5000 µg per plate. Precipitate was observed beginning at 3333 or at 5000 µg per plate. Based on the findings of the preliminary toxicity test, the maximum dose plated in the mutagenicity test was 5000 µg per plate. In the mutagenicity test, the maximum dose tested was 5000 µg per plate; this dose was achieved using a concentration of 100 mg/mL. The test substance solution was clear at this concentration. The dose levels tested were 75, 200, 600, 1800 and 5000 µg per plate. Toxicity was observed with some conditions beginning at 1800 or at 5000 µg per plate. Precipitate was observed at 5000 µg per plate with most test conditions. Low DCPD Resin Oil did not induce a dose-related or 2.0-fold or 3.0-fold increase in the number of revertant colonies in any *Salmonella* strain or in *E. coli* WP2 *uvr*A ±S9. The vehicle controls were acceptable, and the positive control compounds responded appropriately.

Conclusions	Low DCPD Resin Oil did not induce a significant increase in revertant colonies in *Salmonella* strains or in *E. coli* WP2 *uvr*A with or without rat liver metabolic activation at any dose level and is not considered a mutagen in this test system.
Data Quality *Reliabilities*	(1) Reliable without restrictions
Reference	Wagner, V.O. and Hines, R.M. 2003. Low DCPD Resin Oil: Bacterial Reverse Mutation Test. AA75HB.502.BTL. Unpublished Report (DuPont-12984)
Other *Last changed*	16 June 2004

APPENDIX D

Characterization of Low Dicyclopentadiene Resin Oil (Low DCPD Resin Oil)

Supplemental Information for the Test Substance Characterization

1. Page 57: Analytical method provided by the sponsor. Inserted as received from the sponsor (17 pages).
2. Page 74: Table 1. Composition of Low DCPD Resin Oil Test Substance (H-25429, inserted as received from the sponsor).
3. Page 76: Figure 1. Chromatogram of Low DCPD Resin Oil Test Substance (H-25429, inserted as received from the sponsor, 3 pages).
4. Page 79: Analytical method developed at Haskell Laboratory.
5. Page 80: Figure 2. Representative Chromatogram of Low DCPD Resin Oil Test Substance (H-25429) analyzed at Haskell Laboratory.
6. Page 82: Table 2D. Composition of Selected Components of Low DCPD Resin Oil Test Substance.

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MANUAL ANALYTICAL PROCEDURE MANUAL		REVISION DATE APRIL 1995
DOCUMENT TITLE REACTIVES IN AROMATIC RESIN FEEDSTOCK BY GAS CHROMATOGRAPHY		
DOCUMENT NUMBER CHO-LYON-5852 M	DOCUMENT AUTHOR K. W. LOVELL	APPROVERS SIGNATURE

PURPOSE

This procedure describes the GC method for determining reactive content in Lyondell Resin Oil.

SCOPE

Lyondell Resin Oil is analyzed using an instrument equipped with a splitter assembly, flame ionization detector and a 100 meter x 0.32 m ID x 1 micron thickness methyl silicone capillary column. Nelson Analytical software integrates and translates data which is transferred via computer link to the operating units. The dynamic range for this analysis is 0.0005 wt% to 50.0 wt%.

REFERENCES/DEFINITIONS

1. Reference: RJH-01-85, RJH-05-85, RJH-06-85, RJH-07-85, RJH-10-85, RJH-12-85, RJH-13-85, RJH-14-85, RJH-15-85, RJH-16-85, RJH-17-85, RJH-18-85, RJH-26-85, RJH-27-85, RJH-30-85, RJH-31-85, RJH-07-86, KWL-03-93.
2. Original procedure written by C. M. Copeland and J. D. Winter.
3. Revised by R. J. Haynal in December 1984.
4. Revised in January 1992 by Ken Lovell, A. Bettes, E. Foger, J. A. McCormick, and R. J. Haynal.
5. Revised in June 1993 to convert to ISO 9002 formatting.
6. Revised in April 1995 to update Review Statement.

RESPONSIBILITY

The area Technician is responsible for ensuring procedure is followed.

QUALITY CRITICAL

This procedure is Quality Critical because it is used for product certification and by various units in process control.

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HSE CRITICAL

Use care when handling samples. Follow all laboratory safety procedures. Minimum safety protection requirements are lab coat, safety glasses and gloves. Do not inhale vapors or dust and avoid skin contact or ingestion. For additional information refer to the MSDS No.'s provided below.

Benzene	MSDS No. D-AP7594
Hydrogen	MSDS No. D-AP1139
Lyondell Resin Oil	MSDS No. D-AP1446 (LRO-90)

REVIEW

This procedure will be reviewed for accuracy and completeness at least every three years by the Analytical Group.

TRAINING

Training on this procedure will be accomplished during the initial training of employees. In the event of a new procedure, or an existent procedure being changed to different work position, follow-up training will be administered to all affected personnel. The training will be performed by a trainer of the procedure and/or testing position. Before the procedure may be actually used in the production process, the employee must successfully complete the training to meet the Position Verification Guidelines.

PROCEDURE/POLICY

1. Varian 3400 Gas Chromatograph or equivalent:
   a. Flame Ionization Detector
   b. Injector with Varian 1075 Splitter Assembly.
   c. 100M x 0.32 mm ID x 1 micron film thickness
      (Supplied by Supelco Inc./Reference Lot #C217402)
   d. Fritted injection liners - Varian Part #16-000830-1
2. Hamilton syringe - 1.0µL size.
3. Nelson Analytical Interface and Associated software.

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1. Temperature programming

   Initial column temperature 85°C

   Initial hold time 63 min

Prgm	Final Temp	Rate	Hold Time/min	Total Time
1	115°C	3.0°	0/0	73
2	180°C	15.0°		
10.0	87.33			
3	230°C	5.0	10.0	107.33

2. Injector Temperature 200°C
3. Detector Temperature 220°C
4. Flow Parameters
   a. linear viscosity 23.0 cm/sec @ 100°C
   b. splitter exit 100 mL/min
   c. make-up 30 mL/min
5. Flame gases
   a. air 360 mL/min
   b. hydrogen 30 mL/min
6. Miscellaneous
   a. detector range 10^{-11}
   b. attenuation 16
   c. sample size 0.3µL (2,000,000 area counts)

1. Chromatographic Grade Helium (ALPHAGAZ)
2. Chromatographic Grade Hydrogen (ALPHAGAZ)
3. Chromatographic Grade Air

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[redacted]

See CHO-LAB-0604 for calibration procedure.

[redacted]

1. Samples are received in a glass do-pack container. Use proper hand protection when handling and injecting sample.
2. Download correct Nelson chromatography method and identify sample.
3. Activate the proper instrument method.
4. Prepare syringe by flushing sample several times then use appropriate sample size to achieve 3 million area counts (0.3µL with 0.1µL of air and wipe needle)
5. When instrument is equilibrated the "Ready" will appear, inject the sample and push start. The GC will auto-start the Nelson Interface.
6. Nelson chromatography will integrate and format for reporting.

[redacted]

All calculations are handled by Nelson Analytical Software.

[redacted]

Log reactives and 2,6 diterr'ary butyl 1 methyl phenol (BHT) as follows:

a.	Benzene	Log to 2 places.
b.	C7 & Lighter	Log all peaks that elute before toluene including benzene and toluene. Log to 2 places.
c.	Named Reactives	Styrene, a-methyl styrene, cis-B-methyl styrene, meta-vinyl-toluene, trans-B-methyl-indene, total methyl styrene dicyclopentadiene, indene, total methyl-indene (which includes methyl-indene4) and all peaks in between. Log to 2 places.
d.	SS Partial Reactives	Par R1, 2, 3, 4, 5, 6, 14, 15, 19, 20 Log to 2 places.
e.	SS Total Reactives	Tot R7, 8, 9, 10, 11, 12, 13, 16, 17, 18. Log to 2 places.
f.	Total Reactives	Named reactives + ss partial reactives + ss total reactives. Log to 2 places.
g.	BHT	Log BHT to 4 places.

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PRECISION

Repeatability and reproducibility are on SOC data collected for Styrene. Indene and Naphthalene and should not exceed 3.0% relative to retention blend value. Total reactive content is used for Marketing sales and should not exceed 1.0% relative to the retention blend value.

APPENDICES

Attachment I	Nelson Method
Attachment II	GC Chromatogram
Attachment III	Precision Data

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ATTACHMENT I

NELSON METHOD

Lyondell Petrochemical Company

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Method file name : Q:AY010
Default Sample Name: 0125|LRO PROD STD
Operator: GC2
Date-time: 03-14-1993 03:37:19 version: 501

ACQUISITION PARAMETERS

SINGLE OR DUAL CHANNEL (1 OR 2)	1
RUN TIME (minutes)	107.00
END TIME FOR PLOTS (default=RUN TIME)	107.00
SOLVENT DELAY TIME (minutes)	0.00
Area Threshold	0.01
MINIMUM PEAK WIDTH (seconds)	5.00
TIME FOR ONE SAMPLE (seconds)	5.00
NUMBER OF REAL TIME CRT PAGES TO PLOT (0 TO 99)	0.30
REAL TIME PLOT FULL SCALE FOR CH.0 (millivolts)	0.00
REAL TIME FULL SCALE FOR CH.1 (millivolts)	1.00
HARD COPY REAL TIME PLOT	200.00
AUTO ZERO REAL TIME PLOT	NO
Pre Version 4 method	YE
	NO
RECORD AREA TABLES ON DISK	NO
RECORD RAW DATA	YE
NUMBER OF CRT PAGES FOR REPLOT (1 TO 99)	0
VERTICAL SCALE FACTOR FOR REPLOT (units of largest peak)	1.00
OFFSET FOR THE REPLOT (millivolts)	0.00
PUT NAMES ON REPLOT?	YE
AREA PERCENT REPORT	NO
EXTERNAL STANDARD REPORT	NO
INTERNAL STANDARD REPORT	NO
FINAL REPORT AREA REJECT (microvolt-sec)	0.00
LINK TO USER PROGRAM	YE
FORCE DROP LINE INTEGRATION	NO
FORCE COMMON BASE LINE	NO
FULL SCALE RANGE FOR A.D.C. (3=1VOLT, 1=2VOLT, 0=10VOLT)	0
AREA REJECT FOR REFERENCE PEAKS?	99999.00
% RET TIME WINDOW FOR REFERENCE PEAKS	0.00
RET TIME WINDOW IN SECONDS FOR REF. PEAKS	0.00
AREA OR PEAK HEIGHT QUANTITATION (0 OR 1)	0
GROUP REPORT	NO
NUMBER OF CALIBRATION LEVELS (1 TO 6)	1
LIST COMPONENTS NOT FOUND IN SAMPLE?	YE
INCLUDE UNKNOWN PEAKS IN REPORTS?	YE
UPDATE RESPONSE FACTORS WITH REPLACEMENT (0) OR AVERAGE (1)	1
DEFAULT DILUTION FACTOR	1.00
DEFAULT SAMPLE WEIGHT	1.00
DEFAULT AMOUNT INJECTED	1.00
DEFAULT AMOUNT OF INTERNAL STANDARD	1.00
GPC MW DISTRIBUTION	NO
SIMULATED DISTILLATION REPORT	NO

LINK TO PROGRAM : CUSER
SAVE RAW DATA IN : AY01
Response factor for unknowns= 1
Component Units = WT%
Number of Components = 42

IF LOGO IS NOT IN COLOR, THIS IS NOT A CONTROLLED DOCUMENT.

Lyondell Petrochemical Company

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Method:Q:AY010

No.	Component Name	Ret Time	Tol	Fit Type	Resp Fact	RF #
1.	BENZENE	10.70	0.50	2	1.0000	1
2.	TOLUENE	15.40	0.50	2	1.0000	1
3.	ETHYLBENZENE	23.70	0.50	2	1.0000	1
4.	P+M-XYLENE'S	24.70	0.50	2	1.0000	1
5.	STYRENE	27.30	0.50	2	1.0000	1
6.	O-XYLENE	28.05	0.50	2	1.0000	1
7.	a-M-STYRENE	45.05	0.50	2	1.0000	1
8.	UNK C9 ALKBZ	45.17	0.50	2	1.0000	1
9.	c-B-MS	48.00	0.50	2	1.0000	1
10.	m-V-TOL	48.75	0.50	2	1.0000	1
11.	o-V-TOL	49.15	0.30	2	1.0000	1
12.	PSEUDOCUMENE	49.60	0.20	2	1.0000	1
13.	p-V-TOL	50.00	0.30	2	1.0000	1
14.	tbMS + 123HEMMI	58.20	0.50	2	1.0000	1
15.	DCPD	61.40	0.50	2	1.0000	1
16.	UNK INDAN	61.84	0.50	2	1.0000	1
17.	INDENE	64.63	0.50	2	1.0000	1
18.	SS PAR R1	65.12	0.50	2	1.0000	1
19.	SS PAR R2	65.42	0.50	2	1.0000	1
20.	SS PAR R3	72.93	0.50	2	1.0000	1
21.	SS PAR R4	73.26	0.50	2	1.0000	1
22.	SS PAR R5	73.80	0.50	2	1.0000	1
23.	SS PAR R6	74.10	0.50	2	1.0000	1
24.	SS TOT R7	74.30	0.50	2	1.0000	1
25.	SS TOT R8	75.05	0.50	2	1.0000	1
26.	SS TOT R9	75.25	0.50	2	1.0000	1
27.	SS TOT R10	75.72	0.50	2	1.0000	1
28.	SS TOT R11	75.90	0.50	2	1.0000	1
29.	SS TOT R12	76.23	0.50	2	1.0000	1
30.	SS TOT R13	76.95	0.50	2	1.0000	1
31.	SS PAR R14	77.60	0.50	2	1.0000	1
32.	SS PAR R15	77.80	0.50	2	1.0000	1
33.	SS TOT R16	78.12	0.50	2	1.0000	1
34.	SS TOT R17	78.45	0.50	2	1.0000	1
35.	SS TOT R18	78.64	0.50	2	1.0000	1
36.	SSPAR R19/20	79.30	0.50	2	1.0000	1
37.	M-INDEN 1	79.90	0.50	2	1.0000	1
38.	M-INDEN 2	80.32	0.50	2	1.0000	1
39.	M-INDEN 3	80.44	0.50	2	1.0000	1
40.	M-INDEN 4	80.90	0.50	2	1.0000	1
41.	NAPHTHALENE	82.34	0.50	2	1.0000	1
42.	BHT	100.40	0.20	2	1.0600	1

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Lyondell Petrochemical Company

DOCUMENT NUMBER	REVISION NUMBER
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Method: Q:AY010

Event#	Time	Event
1	24.50	EPD-
2	25.05	EPD+
3	57.28	EPD-
4	58.79	EPD+

Event Explanations

1 to 8	(C/O)	relay (Close/Open)
AT	(D/H)	Area Threshold (Double/Halve)
FBA		Force Baseline at All peak starts
BF	(D/H)	Bunching Factor (Double/Halve)
DP	(+/-)	Force Dropline integration (on/off)
FB		Force Baseline at peak start
COM		Common Baseline for next Peaks
CBT	(+/-)	Common Baseline Test (on/off)
PEN	(+/-)	End peaks on Baseline Penetration (o
EPD	(+/-)	End-of-Peak Detection (on/off)
END		End Peak at this Time
EXP	(+/-)	Expo Skim (on/off)
HF	(+/-)	Project Horizontal baseline Forward
HR		Project Horizontal baseline Rearward
NEG	(+/-)	Negative Peak Detection (on/off)
NT	(D/H)	Noise Threshold (Double/Halve)
PD	(+/-)	Peak Detection (on/off)
SKIM		Force straight line peak skim
SPT		Split Peak at this Time
VI		Read vial number after injection

Examples: 1C = relay #1 Closed EXP+ = Expo Skim

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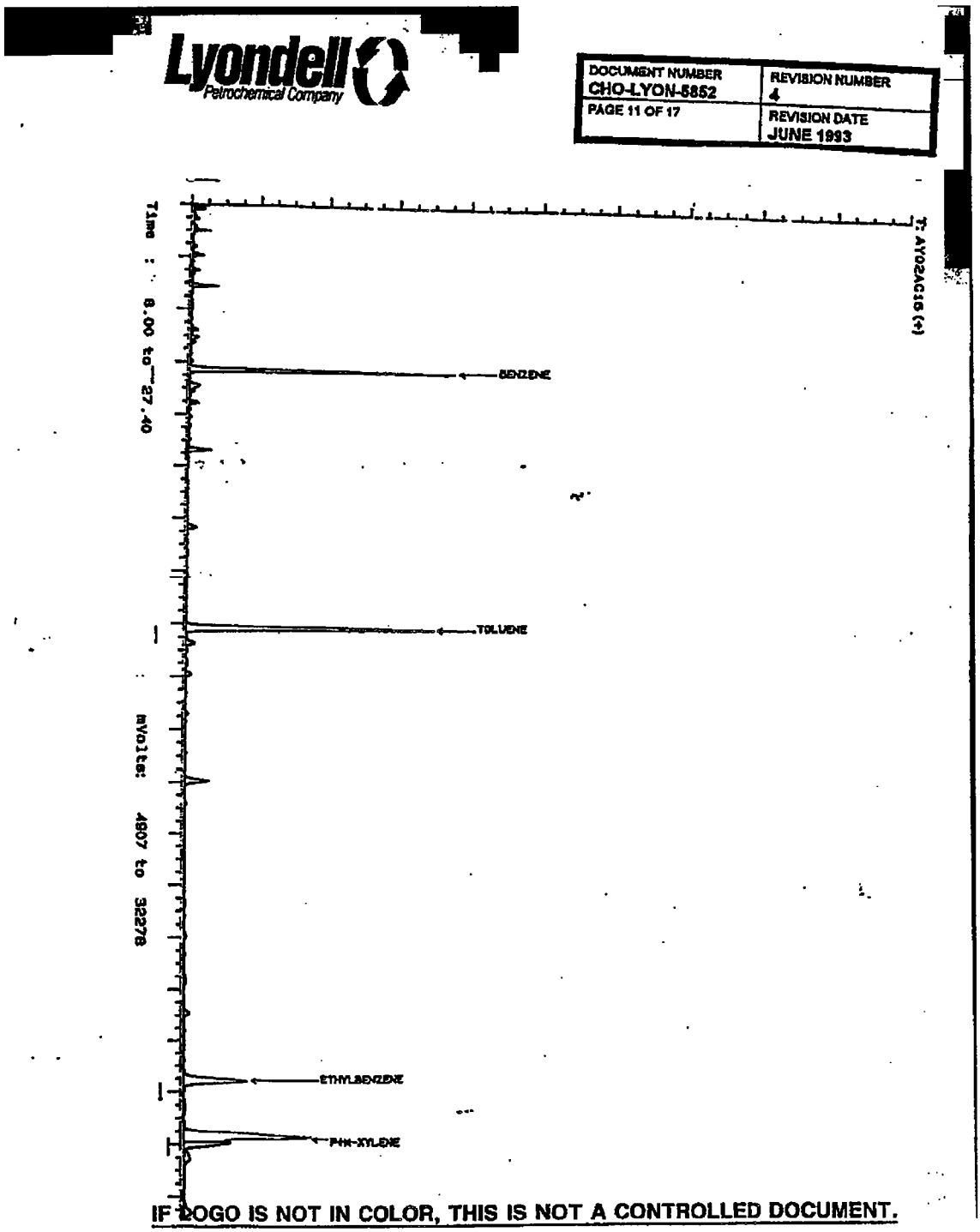
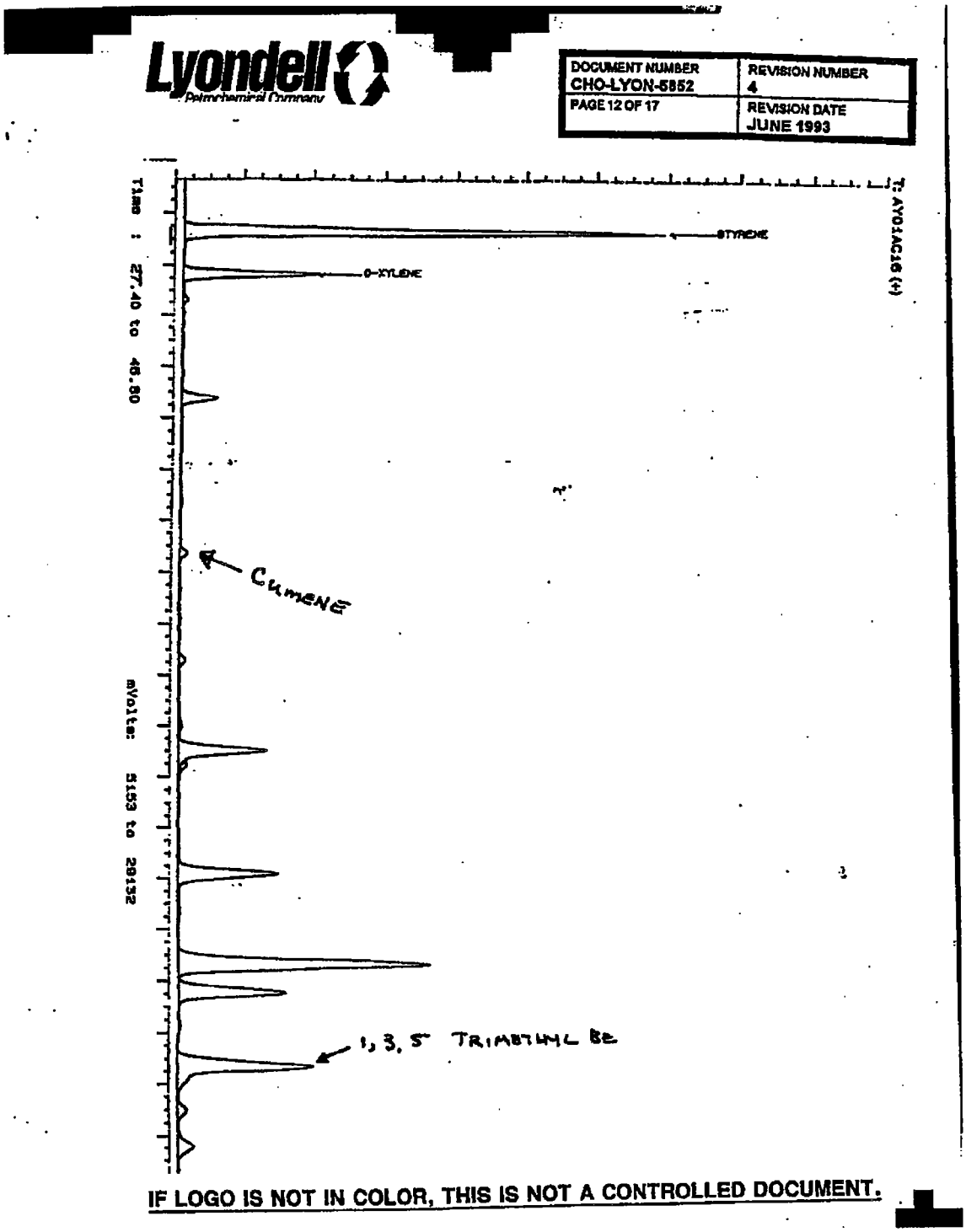
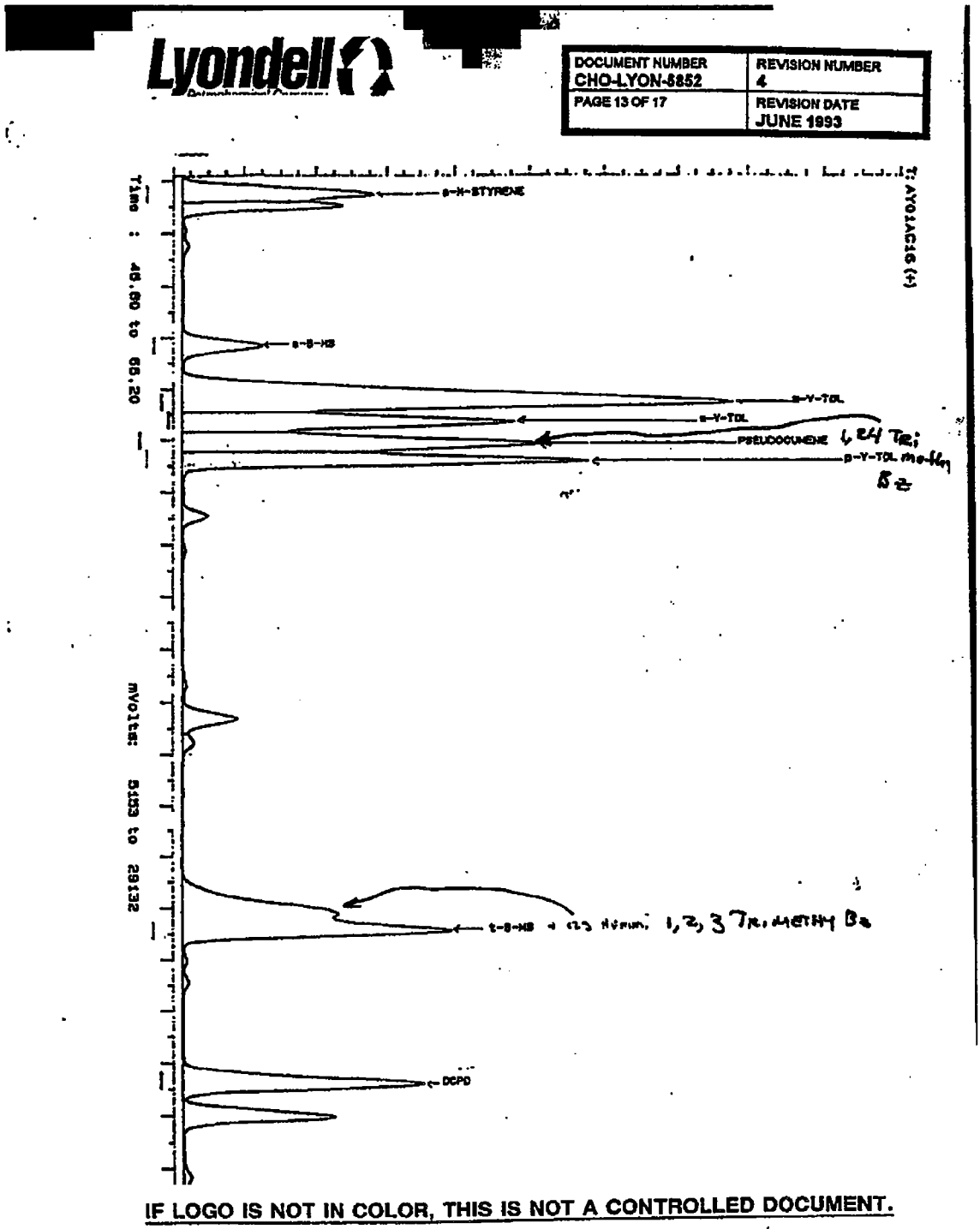
ATTACHMENT II

GC CHROMATOGRAM

IF LOGO IS NOT IN COLOR, THIS IS NOT A CONTROLLED DOCUMENT.


Lyondell
Petrochemical Company
DOCUMENT NUMBER
CHO-LYON-5852
REVISION NUMBER
4
PAGE 11 OF 17
REVISION DATE
JUNE 1993
Time : 8.00 to 27.40
T: AY02AG16 (+)
BENZENE
TOLUENE
mVolts: 4807 to 32278
ETHYLBENZENE
PAR-XYLENE
IF LOGO IS NOT IN COLOR, THIS IS NOT A CONTROLLED DOCUMENT.


Lyondell
Petrochemical Company
DOCUMENT NUMBER
CHO-LYON-5852
REVISION NUMBER
4
PAGE 12 OF 17
REVISION DATE
JUNE 1993
Time : 27.40 to 48.80
mVolts: 5153 to 28132
STYRENE
O-XYLENE
CUMENE
1, 3, 5 TRIMETHYL BE
T: AY01AC16 (+)
IF LOGO IS NOT IN COLOR, THIS IS NOT A CONTROLLED DOCUMENT.



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DOCUMENT NUMBER	REVISION NUMBER
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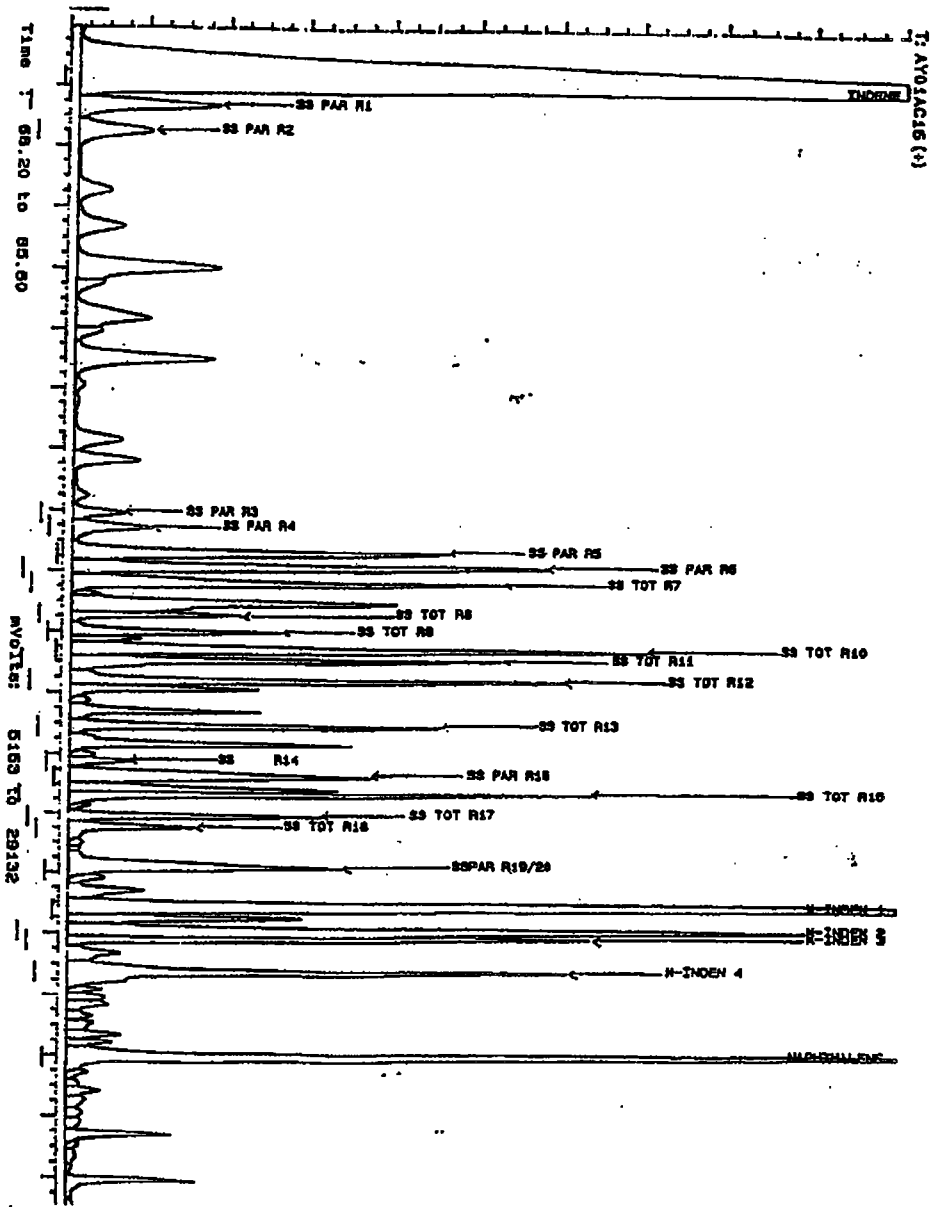

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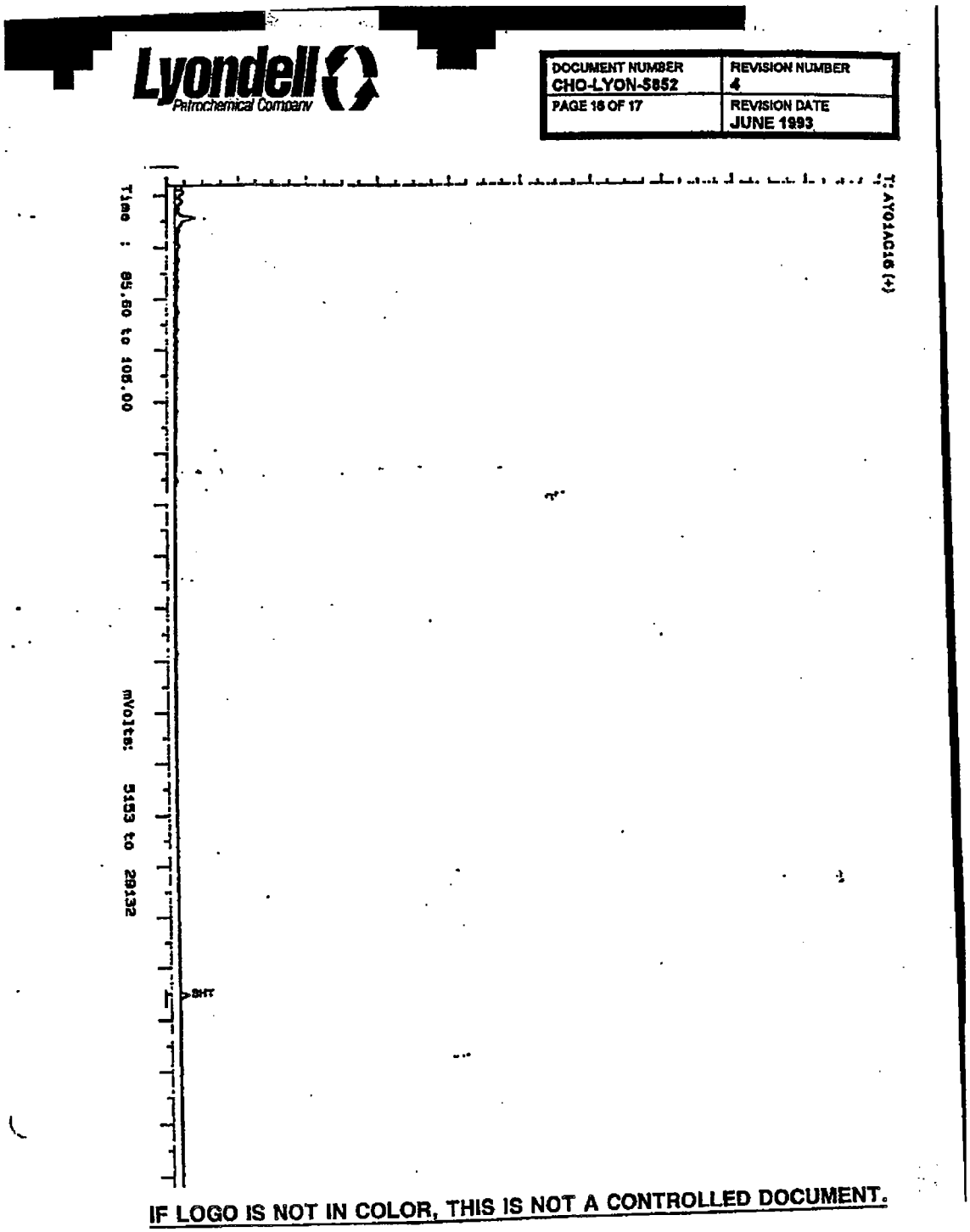
ATTACHMENT III
PRECISION DATA

IF LOGO IS NOT IN COLOR, THIS IS NOT A CONTROLLED DOCUMENT.


Lyondell
Petrochemical Company
DOCUMENT NUMBER
CHO-LYON-5852
REVISION NUMBER
4
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REVISION DATE
JUNE 1993
Time : 85.60 to 105.00
mVolts: 5153 to 28132
T: AY01AC15 (+)
SHT
IF LOGO IS NOT IN COLOR, THIS IS NOT A CONTROLLED DOCUMENT.

[redacted] Lyondell Petrochemical Company

DOCUMENT NUMBER CHO-LYON-5852	REVISION NUMBER 4
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Component	Styrene	Indene	Naphthalene	Total Reactives
	3.98	13.29	4.74	66.43
	3.93	13.11	4.71	66.41
	4.26	13.08	4.87	65.32
	3.98	13.16	4.71	66.31
	3.98	13.11	4.68	66.35
	4.05	13.25	4.56	66.37
	4.14	13.45	4.68	66.69
	3.76	12.98	4.83	66.35
	4.04	13.29	4.68	66.65
	3.84	13.03	4.83	66.05
	3.95	13.22	4.75	66.63
	4.01	13.14	4.68	66.08
	4.08	13.20	4.63	66.36
	4.01	13.22	4.74	66.51
	3.98	13.20	4.75	66.52
	3.99	13.19	4.72	66.49
	3.94	13.10	4.79	66.47
	3.94	13.14	4.79	66.43
	3.77	12.83	4.83	66.06
	3.98	13.10	4.72	66.08
	4.11	13.27	4.71	66.65
Mean wt%	3.98	13.16	4.73	66.34
SD	0.11	0.12	0.07	0.30
Rel SD %	2.83	0.88	1.55	0.46

IF LOGO IS NOT IN COLOR, THIS IS NOT A CONTROLLED DOCUMENT.

Table 1

Manufacturer's characterization of the "Low DCPD Resin Oil" test substance that was submitted for testing in the American Chemistry Council Olefins Panel's HPV program – for the Resin Oils and Cyclodiene Dimer Concentrates Category. The test material was sent on about February 10, 2003, to the EMBSI and Haskell Labs (received on February 27, 2003).

Low DCPD Resin Oil HPV SAMPLE 15:15 2/10/2003

Determinant	Result	Units
BENZENE	0.0005	WT%
C1-C7	0	WT%
TOLUENE	0	WT%
ETHYLBENZENE	0.01	WT%
P+M-XYLENE'S	0.11	WT%
STYRENE	0.52	WT%
O-XYLENE	0.18	WT%
1,3,5-TRIMETHYLBENZENE	1.8	WT%
alpha-METHYL STYRENE	1.1	WT%
cis-beta-METHYL STYRENE	0.55	WT%
meta-VINYL TOLUENE	10.04	WT%
ortho-VINYL TOLUENE	2.98	WT%
PSEUDOCUMENE	6.45	WT%
para-VINYL TOLUENE	4.27	WT%
trans- beta METHYL STYRENE	1.06	WT%
123HEMMI	0.92	WT%
DCPD	0.68	WT%
INDENE	13.68	WT%
METHYL INDENES (TOTAL)	8.35	WT%
NAPHTHALENE	1.47	WT%
BHT	0.0074	WT%
Total	54.1705	

Additional Comment: The manufacturer's characterization of the Low DCPD Resin Oil test substance identifies and quantifies the major resin-forming components in the complex mixture. The identified components are primarily aromatic hydrocarbons, with a carbon range of C8 through C10. These components account for about 54 wt% of the mixture. The balance of the composition is expected to consist primarily of other C8 through C10

hydrocarbons (mainly aromatics and lesser amounts of paraffins and olefins). The chromatogram generated by the manufacturer's GC analysis of the test substance indicates more than 100 peaks (corresponding to unidentified hydrocarbon species in the mixture) that have retention times similar to the identified aromatics. The individual unknown peaks are quantified on the chromatogram and the individual values range form less than 0.01 wt % to as much as about 2.2 wt%. The Low DCPD Resin Oil is produced as a distillate fraction with a boiling range of about 335 to 410 degrees F.

Figure 1

Manufacturer-supplied chromatogram of the " Low DCPD Resin Oil" test substance that was submitted for testing in the American Chemistry Council Olefins Panel's HPV program – for the Resin Oils and Cyclodiene Dimer Concentrates Category. The test material was sent on about February 10, 2003, to the EMBSI and Haskell Labs (received on February 27, 2003).

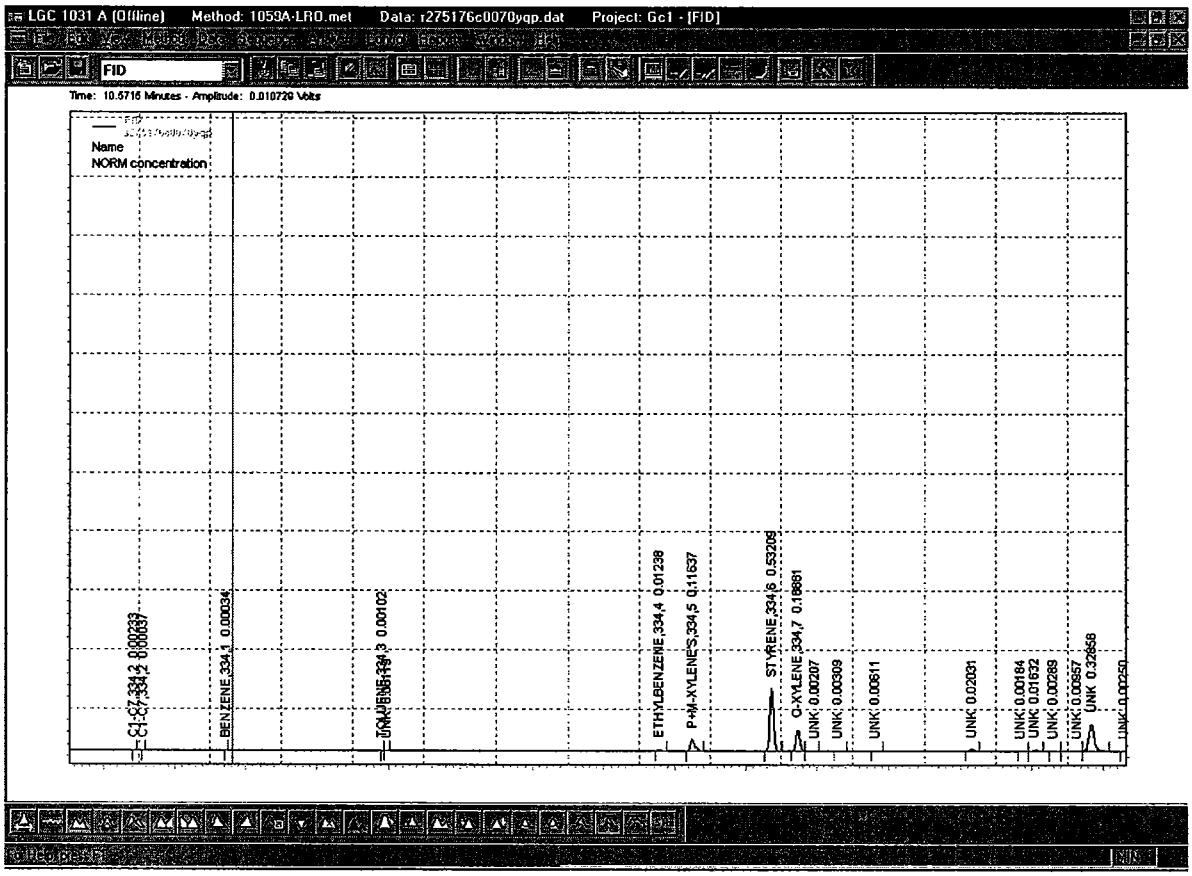


Figure 1 (continued)

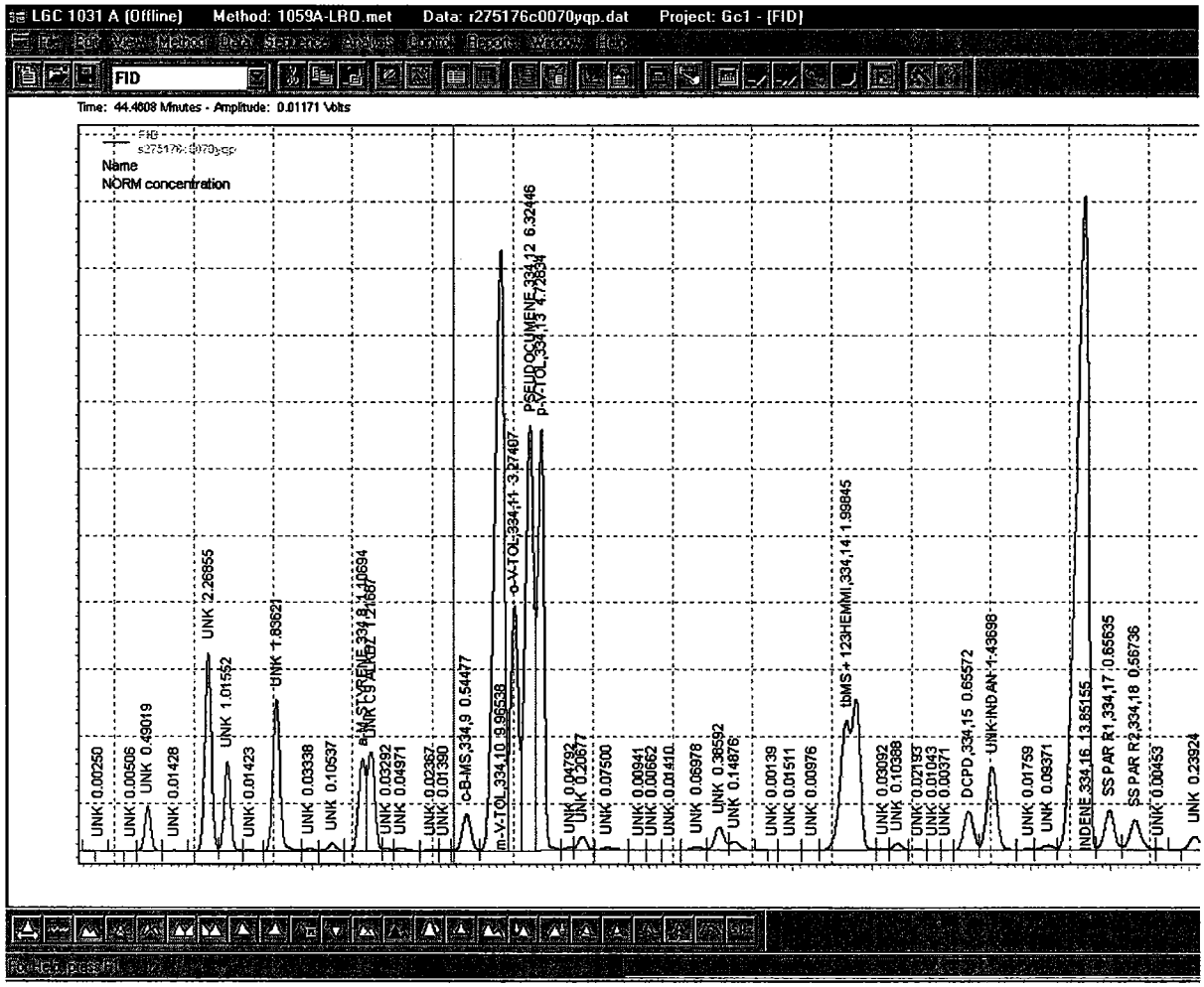
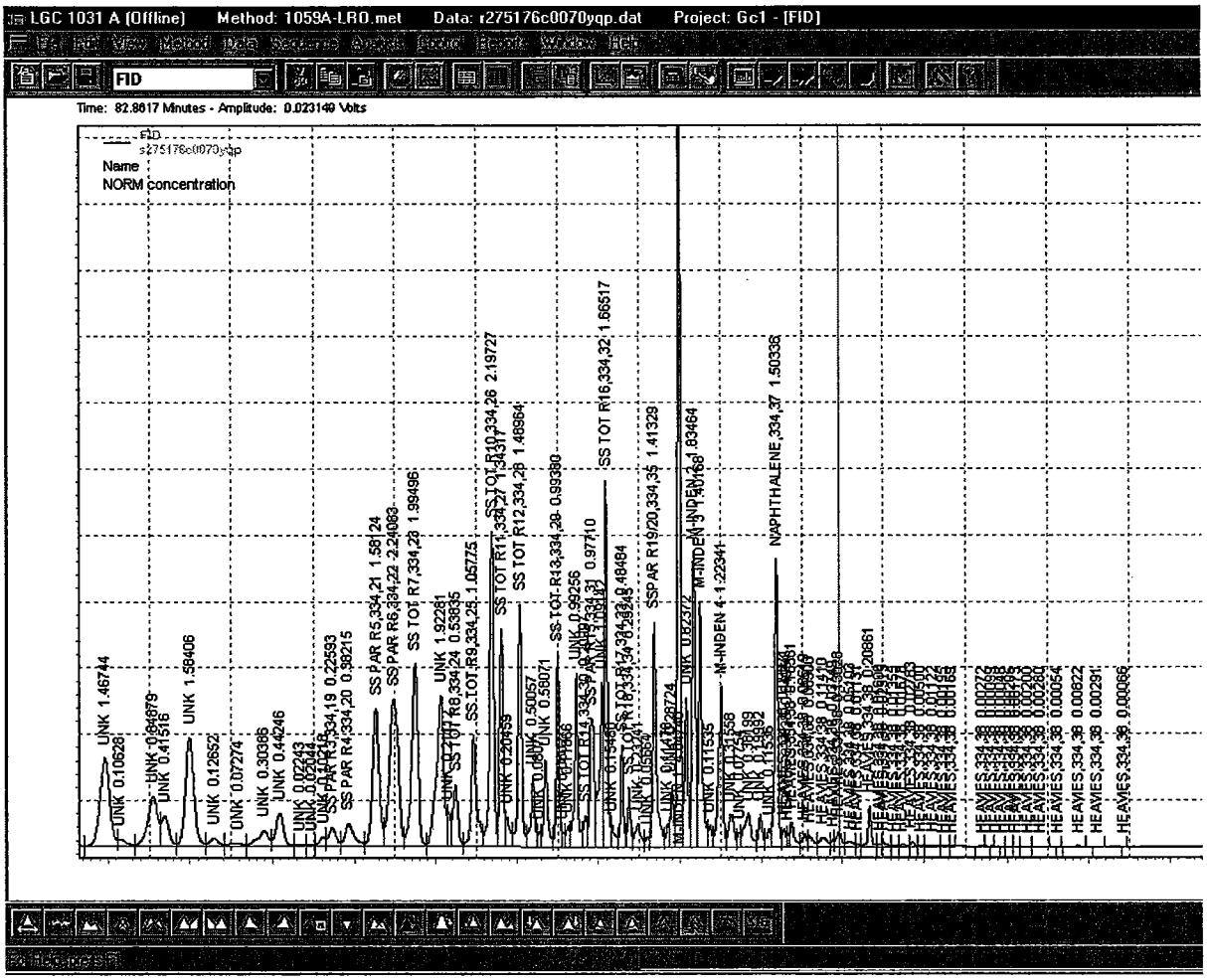


Figure 1 (continued)



Analytical method used at Haskell Laboratory:

The method used for analysis of Low DCPD Resin Oil was based on the method provided by the sponsor (see above).

The instrument and the experimental conditions pertaining to the GC analysis are summarized below:

Instrument:	Hewlett Packard HP6890 gas chromatograph with 7683 autosampler
GC Column:	Supelco SPB-1, 100 m length, 0.23 mm internal diameter, 1.0 µm film thickness
Carrier Gas:	Helium
Injector Temperature:	200°C
Pressure:	24.9 psi
Split Ratio:	52.1:1
Wash Solvent A:	Chloroform
Syringe Size:	5 µL
Injection Volume:	0.1 µL
Carrier Gas Flow Rate:	1.8 mL/min, constant flow mode
Initial Temperature:	85°C, hold for 63 minutes, then increase at 3°C/min to Final Temperature 1
Final Temperature 1:	115°C, increase at 15°C/min to Final Temperature 2
Final Temperature 2:	230°C, hold at Final Temperature 2 for 2 minutes
Run Time:	99.33 min
Detector:	Flame Ionization Detector (FID)
Detector Temperature:	220°C
Hydrogen Flow:	40 mL/min
Air Flow:	450 mL/min
Makeup Gas Type:	Helium

Figure 2

Representative chromatogram of Low DCPD Resin Oil Test Substance (in order to avoid overlapping labels, not all the peaks were annotated).

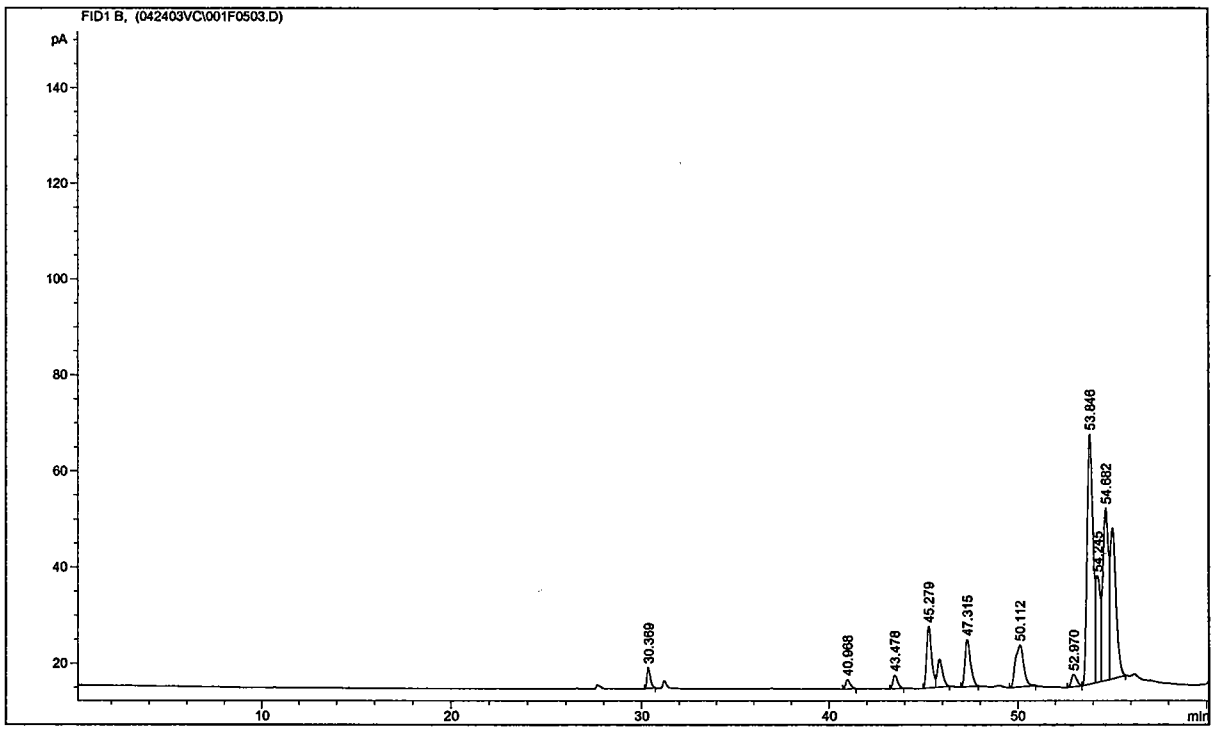


Figure 2 (continued)

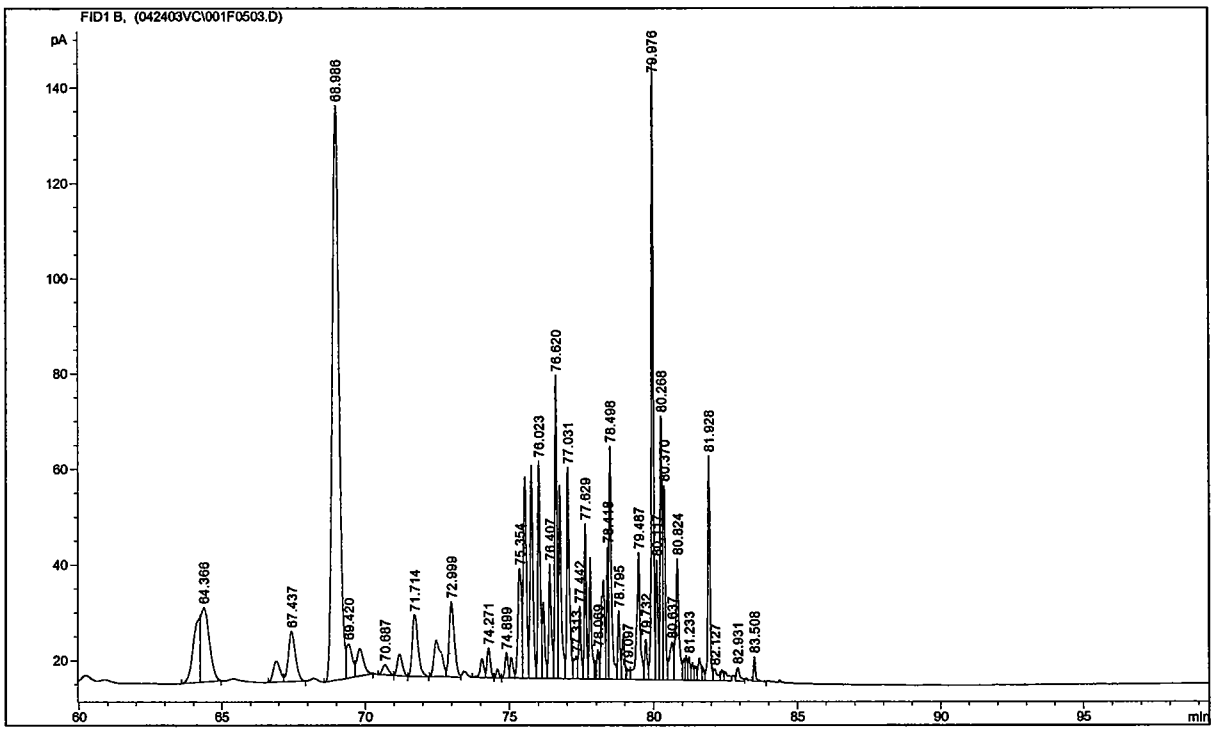


Table 2D

Retention times and composition of representative components in the chromatogram in Figure 2.

Retention Time (min)	Component Name[a]	Area %	Supplier's Composition (%)
53.846	m-vinyltoluene	8.52	10.04
55.682	1,2,4-trimethylbenzene	5.77	6.45
55.026	p-vinyltoluene	5.08	4.27
68.986	indene	14.38	13.68

[a] Components identified based on Sponsor-provided gas chromatographic profile